Abstract

Calpains have been previously shown to regulate AMPA receptor properties by producing partial truncation of the C-terminal domains of several receptor subunits. We now report that NMDA receptor subunits, in particular NR2 subunits, are also subjected to calpain-mediated truncation. Treatment of synaptic membranes with calpain I resulted in truncation of both NR1 and NR2 subunits, with the appearance of NR2 species with lower mol.wt. than native subunits, but still recognized by antibodies directed at the C-terminal domain. This treatment did not modify the binding of several ligands of the NMDA receptors, such as glutamate, glycine or TCP. Incubation of thin frozen-thawed brain sections with calcium resulted in calpain-mediated selective degradation of NR2 subunits, as truncation into smaller fragments was totally blocked by calpain inhibitors. Under the same conditions, TCP binding to sections was decreased by about 50%, an effect also blocked by calpain inhibitors. Treatment of hippocampal slices in culture with the excitotoxin, kainic acid, also produced calpain-mediated truncation of the C-terminal domain of NR2 but not NR1 subunits of the NMDA receptors. The results indicate that calpain activation produces several modifications of NMDA receptors, including the truncation of the C-terminal domain of NR2 subunits, and changes in channel binding properties. They suggest that calpain-mediated regulation of NMDA receptors might represent a feed-back regulation of the receptors which could be used to limit receptor activation. © 1998 Elsevier Science B.V.

Keywords: Calpain; Glutamate; Receptor; Plasticity; Calcium; Hippocampus

1. Introduction

N-methyl-D-aspartate (NMDA) receptors play a central role in a variety of physiological as well as pathological phenomena in the central nervous system. In particular, activation of the NMDA receptors is necessary and sufficient to induce a long-lasting increase in synaptic transmission in several monosynaptic excitatory pathways, a phenomenon referred to as long-term potentiation (LTP) [7,15,25,26]. In addition, NMDA receptor stimulation is also critically involved in activity-dependent modifications of neural connections during the developmental period [8]. Finally, prolonged stimulation of NMDA receptors has been shown to lead to neuronal injury and is possibly involved in neuronal degeneration taking place as a result of ischemia/hypoxia, hypoglycemia and several neurodegenerative diseases [5,6,9,23]. A key feature of the NMDA receptors underlying this multiplicity of function is the calcium permeability of its associated ionic channel [22]. NMDA receptors are heteromeric proteins comprising two families of subunits, NR1 and NR2 generated from alternative splicing of a single gene or from different but related genes, respectively [11,24]. Although the exact stoichiometry as well as the transmembrane structure of the receptors are still debated, a number of arguments suggests that both NR1 and NR2 subunits exhibit three transmembrane domains with extracellular N-terminal and intracellular C-terminal domains [13,17,18,32]. In particular, the C-terminal domain of the receptors has been shown to be responsible for the anchoring of the receptors to the cell cytoskeleton [12,13,17,18].

NMDA receptor subunits have a significant sequence homology with subunits belonging to another family of ionotropic glutamate receptors, the AMPA receptors. We recently found that AMPA receptor subunits can be proteolytically truncated in their C-terminal domains as a result of the activation of the calcium-dependent protease, calpain [1,3,4]. By using antibodies directed against the N-terminal and C-terminal domains of the GluR1 subunits, we provided evidence for the existence of at least two
cleavage sites for calpain in the C-terminal domain, generating two truncated species of GluR1, one lacking a very small fragment of the carboxyl end and the other with a mol. wt., 7 kDa smaller than the original subunit [1,3]. Although the functional significance of the modifications of the receptors resulting from cleavage at either sites remains unknown, we showed that cleavage occurs in situ following NMDA receptor activation and is associated with increased responsiveness of the receptors [14,27]. Furthermore, it was recently reported that the L-type calcium channels are also proteolytically truncated in their C-terminal domain as a result of calpain activation, suggesting that calpain-mediated truncation of C-terminal domains of membrane receptor/channels might be a feature shared by a number of receptor/channels [16]. It was therefore, of interest to evaluate the possibility that NMDA receptor subunits could also be proteolytically modified as a result of calpain activation. In this report, we show that NR2 and NR1 subunits are subjected to calpain-mediated proteolysis. Furthermore, we also present evidence that in situ calpain activation produces NMDA receptors with altered channel properties. Our results raise the possibility that NMDA receptors are self-regulated through calpain-mediated truncation of the C-terminal domain of its subunits.

2. Materials and methods

2.1. Preparation of synaptic membranes

Synaptic membranes were prepared from the telencephalon of adult Sprague–Dawley rats (200–250 g) as described by Massicotte et al. [21] with minor modifications, which consisted in including 0.1 mM leupeptin in the initial steps of membrane preparations. Following hypotonic shock, the membrane pellet was resuspended twice in 50 mM Tris–acetate, pH 7.4 buffer containing 0.1 mM EGTA (TA buffer), and centrifuged at 48,000 × g for 20 min. After the final centrifugation and resuspension in TA buffer, membrane fractions were stored in aliquots at −70°C until used. Membranes were incubated at 35°C with either calcium alone or calcium with purified calpain I (120 units/mg protein; Calbiochem, La Jolla, CA) at the indicated concentrations and for periods of time indicated in figure legends.

2.2. Binding assays in synaptic membranes

Binding experiments were performed as previously described [21]. Briefly, synaptic membranes were centrifuged at 48,000 × g for 20 min following pretreatment with calcium or purified calpain I (Calbiochem). For TCP binding, synaptic membranes were then resuspended in 5 mM HEPES buffer pH 7.4 containing 100 μM EGTA. TCP binding was measured by incubating synaptic membranes with 10 nM [3H]-TCP (50.4 Ci/mmol; NEN, Boston), generally in the presence of 10 μM glutamate and 10 μM glycine (to maximally activate the receptor/channel) at room temperature for 40 min [20]. In some experiments, 10 nM [3H]-MK-801 (20.0 Ci/mmol; NEN) was used instead of [3H]-TCP. Glycine and glutamate binding to NMDA receptors were determined as described previously [21]. Protein concentration of samples was determined using the BioRad protein assay.

2.3. Calcium treatment of brain sections

Adult Sprague–Dawley rats were killed by decapitation following anesthesia and brains were rapidly removed, frozen in 2-methylbutane at −40°C and stored at −70°C. Serial frontal sections (20 μm thick) were cut on a cryostat, thaw-mounted onto chrome–alum gelatin-coated slides, and kept at −70°C until used (less than 1 week). Adjacent sections were thawed at room temperature and preincubated in Tris–acetate buffer (100 mM, pH 7.4) containing 0.1 mM EGTA for 2–60 min at either 0–4°C or 35°C with or without calcium acetate (0.4–4.0 mM). Leupeptin, calpeptin (Sigma) or calpain inhibitor I (Boehringer Mannheim, dissolved in 0.1% dimethyl sulfoxide) were also used at a concentration of 0.1–0.4 mM. At the end of the incubation, sections were removed from slides, homogenized in 10 mM Tris–HCl buffer (pH 7.6) containing 0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, 0.1 mM leupeptin, and TPCK (1 μg/ml), and further processed for Western blots as described below, while other sections were processed for ligand binding.

2.4. Preparation of hippocampal slice cultures

Organotypic hippocampal cultures were prepared using the technique of Stoppani et al. [31] as previously described [14]. Hippocampal cultures were incubated at 35°C with a 5% CO2-enriched atmosphere and fed twice weekly until use.

2.5. KA treatment of hippocampal slice cultures

After 7–14 days in vitro, hippocampal cultures were incubated with growth medium without serum, with or without the addition of calpeptin (100 μM in 0.05% DMSO, a generous gift from Dr. Kiso, Suntory) for 3 h at 35°C, followed by an exposure to KA (50 μM in 0.9% saline, Sigma Chemical, St. Louis, MO) for 3 h in the presence or absence of calpeptin in exposure medium (serum-free growth medium containing 4 mM CaCl2). Control cultures were incubated under the same conditions with the omission of calpeptin and KA. KA treatment was stopped by washing with serum-free growth medium, and
Fig. 1. Effects of calpain treatment of synaptic membranes on NR1 and NR2 subunits of NMDA receptors. Synaptic membranes from rat forebrain were prepared as described in Section 2 and incubated at 35°C for 30 min in the absence of calcium (Cont), in the presence of 2 mM calcium (Ca²⁺), 2 mM calcium plus calpain I (32 μg/ml) (Calpain I), leupeptin (0.1 mM) (Leu), 2 mM calcium plus 0.1 mM leupeptin (Ca²⁺/Leu) or 2 mM calcium plus calpain I plus leupeptin (Calpain I/Leu). At the end of the incubation, aliquots were processed for Western blotting and the blots stained with antibodies against the C-terminal domains of NR1 (A) or NR2A/B (B). In A, the bars on the left represent mol.wt. markers of 118 and 86 kDa, respectively. Arrows point to breakdown products still recognized by the antibodies observed following treatment with calcium at 60 and 52 kDa. The experiment was repeated three times with identical results.

Hippocampal slices were processed either immediately (3 h) or 24 h (24 h) after KA treatment for Western blots. No cytotoxic effect was detected by visual examination of propidium iodide (PI) (0.46 μg/ml; Sigma Chemical) uptake and enzymatic measurement of lactate dehydrogenase release after the application of either vehicle alone (0.05% DMSO) or calpeptin (100 μM in 0.05% DMSO) up to 3 h after KA treatment.

Fig. 2. Time-course for the effects of calpain treatment of synaptic membranes on NR1 and NR2 subunits of NMDA receptors. Same legend as under Fig. 1, except that the duration of treatment with calcium plus calpain was varied from 5 to 30 min. Treatments with calcium alone (Ca²⁺), calcium plus leupeptin (Ca²⁺/Leu) or calcium plus calpain plus leupeptin (Calpain/Leu) were for 30 min. The blots were scanned and the amounts of the 110 kDa (NR1) and the 170 kDa (NR2A/B) bands were determined and expressed as percent of control. Results are means of 2–3 experiments.
Fig. 3. Effects of calcium treatment of tissue sections on NR1 and NR2 subunits of NMDA receptors. Frozen-thawed (20 μm) tissue sections were incubated at 35°C for 60 min in the presence of 0 (Cont), 0.2, 0.4, 1, 2 or 4 mM calcium. Tissue was then collected and processed for Western blots using antibodies against the C-terminal domain of NR1 and NR2A/B subunits of the NMDA receptors. Top panels: representative blots from one experiment. Bottom panels: quantitative analysis of blots; the amounts of the 110 kDa (NR1) and the 170 kDa (NR2A/B) bands were determined and expressed as percent of control. Results are means ± S.E.M. of 3–4 experiments (* p < 0.05; ANOVA followed by post-hoc analysis with the LSD test).

2.6. Western blots

Following incubation, synaptic membranes were diluted with equal amount of 2 × sample buffer (1 × sample buffer consists of 2% sodium dodecyl sulphate (SDS), 50 mM Tris–HCl (pH 6.8), 2.5% 2-mercaptoethanol, 10% glycerol and 0.1% Bromophenol Blue). Samples were boiled at 100°C for 5 min, and membrane proteins (30–40 μg per lane) were subjected to SDS–PAGE performed according to the method of Laemmli [19] using 8% polyacrylamide gels; proteins were then transferred onto nitrocellulose membranes as described by Towbin et al. [33] and membranes were processed for immunostaining as previously described [1,3] with antibodies against NR1 or NR2 (Chemicon, dil. 1:200). In addition, an antibody against a spectrin breakdown product (SBDP) (1:5000; a generous gift from Dr. R. Siman, Cephalon, West Chester, PA) specifically generated by calpain-mediated degradation of spectrin was used to provide a quantitative assessment of calpain activation. Immunoblots were scanned and the digitized images were quantitatively analyzed by densitometry with the ImageQuant program providing peak areas and apparent mol wt.

2.7. Ligand binding in tissue sections

After pretreatment under various conditions, tissue sections were rapidly washed in cold 5 mM HEPES buffer, and incubated at room temperature in the presence of 10 μM glutamate and 10 μM glycine (to maximally activate the receptor/channel) and 10 nM 3H-TCP for 40 min. At the end of incubation, sections were rinsed in HEPES buffer, and were wiped off the slides with a filter paper. Radioactivity was determined by scintillation counting. Non-specific binding was determined by incubating adjacent sections under the same conditions in the presence of 1 mM ketamine.

Fig. 4. Effects of calcium treatment of tissue sections on 3H-TCP binding to the NMDA receptors. Frozen-thawed (20 μm) tissue sections were incubated at 35°C for 60 min in the presence of 0 (Cont), 2 mM calcium (Ca2+), 0.1 mM leupeptin (Leu) or 2 mM calcium plus 0.1 mM leupeptin (Ca2+/Leu). 3H-TCP binding was then determined by incubating tissue sections at room temperature for 45 min in the presence of 10 μM glycine and 10 μM glutamate. Sections were washed and radioactivity determined by liquid scintillation. Non-specific binding was determined as the binding measured in the presence of 1 mM ketamine. Data are expressed as percent of specific binding in control and are means ± S.E.M. of 6–8 experiments (* p < 0.01, Student’s t-test).
3. Results

3.1. Effects of calpain treatment of synaptic membranes on NMDA receptor subunits

Synaptic membranes from rat telencephalon were incubated at 35°C in the presence of calcium alone (2 mM) or with the addition of calpain I. Antibodies recognizing C-terminal domains of NR1 and NR2 were then used to assess the apparent mol.wt. and amount of the receptor subunits in Western blots (Fig. 1). Treatment with calcium alone produced a small calpain activation as indicated by the appearance of SBDP immunoreactive material (not shown), but did not produce significant changes in NR1 and NR2. Addition of calpain I resulted in large accumulation of SBDP and produced significant decrease in the intensity of the bands labeled with NR2 and NR1 antibodies migrating with apparent mol.wt. of 170 and 110 kDa, respectively. In parallel, with the decrease in intensity of the NR2 band, at least two new immunopositive bands appeared with mol.wt. of 60 and 52 kDa. These effects were considerably reduced when calpeptin (100 μM) or calpain inhibitor I (100 μM), two calpain inhibitors, were included in the incubation. To further characterize the effects of calpain treatment of synaptic membranes on NMDA receptor subunits, we incubated membranes with

![Fig. 5. Effects of kainate treatment of cultured hippocampal slices on NR1 subunits of the NMDA receptors. Cultured hippocampal slices were incubated with kainic acid (50 μM) for 3 h and collected immediately (3 h) or 24 h later (24 h) and processed for Western blots using antibodies against the C-terminal domain of NR1 subunits. When present, calpeptin (100 μM) was present for 3 h before as well as during and after KA treatment. Top panels: representative blots from one experiment. Bottom panels: quantitative analysis of blots; the amounts of the 110 kDa (NR1) band were determined and expressed as percent of control. Results are means ± S.E.M. of 3–4 experiments.](image-url)
calpain for various periods of time (Fig. 2). The effects of calpain occurred rapidly as both NR1 and NR2 were decreased by 35 ± 3% and 40 ± 4%, respectively after 5 min of incubation; a maximal decrease of 50 ± 5% and 53 ± 5% was reached by 30 min of incubation for NR1 and NR2, respectively.

To determine potential functional modifications of receptor properties resulting from partial proteolysis, we evaluated the effects of similar treatments of synaptic membranes on the binding of ^3^H-glutamate, ^3^H-glycine and ^3^H-TCP, an activity-dependent ligand for the channel sites of the NMDA receptor. No effect of calcium or calcium plus calpain treatment was found for ^3^H-glutamate binding. Similarly, basal or glutamate and glycine-stimulated ^3^H-TCP binding was not significantly modified following calcium treatment alone, or in the presence of calpain I (data not shown).

### 3.2. Effects of calpain activation in frozen-thawed brain slices on NMDA receptor properties

Frozen-thawed brain sections (20 μm thick) were incubated at 0 or 35°C in the presence or absence of various calcium concentrations for various periods of time. After the incubation, sections were collected, homogenized, and NR1 and NR2 proteins were identified in Western blots. Calcium treatment resulted in calpain activation as indicated by the accumulation of SBDP (not shown). The same treatment had no significant effect on NR1 subunits (Fig. 3). However, under the same conditions, it resulted in a significant decrease in the intensity of the NR2 subunits. A

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**Fig. 6. Effects of kainate treatment of cultured hippocampal slices on NR2 subunits of the NMDA receptors.** Cultured hippocampal slices were incubated with kainic acid (50 μM) for 3 h and collected immediately (3 h) or 24 h later (24 h) and processed for Western blots using antibodies against the C-terminal domain of NR2 subunits. When present, calpeptin (100 μM) was present for 3 h before as well as during and after KA treatment. Top panels: representative blots from one experiment. Bottom panels: quantitative analysis of blots; the amounts of the 170 kDa (NR2) band were determined and expressed as percent of control. Results are means ± S.E.M. of 3–4 experiments.
significant effect of calcium was observed at a calcium concentration of 0.2 mM, and the maximal effect was observed at 1 mM calcium. As the intensity of the band migrating with an apparent mol. wt. of 170 kDa decreased, new immunoreactive bands appeared with mol. wt. of 60 and 52 kDa. No effect of calcium was observed when the incubation was performed at 0°C or in the presence of calpeptin or calpain inhibitor I (not shown).

We also evaluated the effects of calcium treatment on the binding of 3H-glutamate and 3H-TCP to the NMDA receptor channels in tissue sections (Fig. 4). Calcium treatment did not modify 3H-glutamate binding but produced a significant decrease in 3H-TCP binding. This effect was blocked by leupeptin (200 μM) or calpain inhibitor I (100 μM) (not shown).

3.3. Effect of kainic acid treatment of cultured hippocampal slices on NMDA receptor subunits

To determine whether calpain-mediated truncation of NMDA receptor subunits occurs in situ under physiological or pathological conditions, hippocampal slice cultures were subjected to KA exposure (50 μM for 3 h) and homogenized immediately (3 h), or 24 h (24 h) after incubation with serum-free growth medium, and processed for Western blots. KA treatment did not modify the amount of NR1 immunoreactivity at either 3 or 24 h (Fig. 5). In contrast, it did produce a significant decrease in the amount of NR2 subunits both at 3 h and even more so at 24 h (Fig. 6). Furthermore, the decrease in the amount of the 170 kDa band labeled with antibodies against the C-terminal domain of NR2 subunits was associated with an increase in 2 bands migrating with apparent Mₙ of 60 and 52 kDa (Fig. 7). Both effects of KA treatment, i.e., the decrease in the 170 kDa band and the increase in the 60 and 52 kDa bands were completely blocked when calpeptin was present during KA treatment (Fig. 6).

4. Discussion

The present results indicate that NMDA receptor subunits, and in particular NR2, are proteolytically truncated in their C-terminal domains as a result of calpain activation. Results obtained with calpain treatment of membranes showed that both NR1 and NR2 subunits are substrates of calpain. In the case of NR2, calpain-mediated degradation resulted in the formation of degradation products still recognized by antibodies against the C-terminal domain. Moreover, the size of the fragments matched quite well with those of fragments that could be generated should NR2 be cleaved at putative calpain consensus sites located in the long intracellular C-terminal domain (Table 1). In the case of NR1, no fragments with lower molecular size were observed, a result similar to what was observed with GluR1 subunits of the AMPA receptors [1,3,4]. However, it is not clear that calpain-mediated truncation of

<table>
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<th>Sequences</th>
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<th>Size of fragment</th>
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<tr>
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<td>846–847</td>
<td>→ ~ 60 kDa</td>
</tr>
<tr>
<td>(2) …GNLIFY SDN…:</td>
<td>924–925</td>
<td>→ ~ 52 kDa</td>
</tr>
<tr>
<td>(3) …QYRLFY AKHF…:</td>
<td>1168–1169</td>
<td>→ ~ 28 kDa</td>
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Potential cutting sites were determined by comparison of the amino acid sequences of NR2 subunits with preferred sequences for calpain-mediated proteolysis determined with a variety of peptide sequences [10,29].
NR1 in synaptic membranes has real significance as it was not observed following in situ calpain activation resulting from calcium treatment of tissue sections or KA treatment of hippocampal slices in cultures. Results obtained with calcium treatment of brain sections further confirmed the calpain-mediated selective truncation of the C-terminal domain of NR2 subunits, as the calcium-dependent formation of fragments of the receptors with lower mol. wt. was completely blocked by calpain inhibitors. We cannot exclude the existence of additional cleavage sites which could generate even smaller fragments that would not be detected in Western blots. Furthermore, it is not clear that these smaller fragments are stable and that they are not further processed by calpains or other proteases. In fact, close analysis of Fig. 1 indicates that these smaller fragments are much more evident following calcium treatment of membranes (which presumably corresponds to activation of endogenous calpain only) than with calpain treatment, suggesting that they could be further processed by calpain itself.

Our results also indicate that calpain-mediated truncation of the C-terminal domain of NR2 receptors leads to functional modifications of the NMDA receptor/channels. While no change in binding for the agonist glutamate and the co-agonist glycine was observed, truncation produced a large decrease in \(^{3}H\)-TCP or \(^{3}H\) MK-801 (data not shown) binding to the receptor/channel complex. The decrease in binding was observed whether glutamate or glycine or the combination was present suggesting that it is more likely, the configuration of the channel which is altered rather than its activation by agonists. However, it is important to stress that calpain treatment of membranes producing maximal truncation of the receptor subunits did not result in changes in binding of several ligands of the receptors (i.e., glutamate, glycine and TCP). It was only when NR2 subunits were selectively truncated as a result of in situ calpain activation that changes in TCP binding were observed. Whether the decrease in TCP binding is due to the truncation of NR2 subunits or to some other calpain-mediated effect is currently not known. Nor is it clear why NR1 subunits are not truncated following in situ calpain activation. It is conceivable that in situ interactions between NMDA receptors and other proteins protect NR1 subunits from degradation. In particular, the C-terminal domain of NR1 subunits has been shown to bind calmodulin [12], whereas, NR2 subunits bind to the members of the PDZ family of NMDA receptor-anchoring proteins [12,17,18]. Alternatively, the state of phosphorylation of NR1 and NR2 subunits might be quite different between the membrane preparation and the in situ condition, and this could result in a selective protection of NR1 subunits. Further experiments directed at testing the effects of phosphorylation of NR subunits on calpain-mediated truncation should provide interesting information regarding these questions. Also worth mentioning is the fact that a large fraction of NR2 subunits is resistant to calpain-mediated truncation in synaptic membranes as well as in tissue sections. It is possible that different NR2 subunits are differentially susceptible to calpain, although preliminary experiments with antibodies specific for NR2A and NR2B did not reveal any difference for these two subunits.

Although further experiments are needed to establish the functional consequences of calpain-mediated truncation of NR2 subunits, the results of the present study clearly indicate the existence of a new calcium-dependent regulation of NMDA receptors, which could modify the properties of the receptors for the life-time of the receptors. Activation of NMDA receptors under both physiological and pathological conditions has been shown to produce calpain activation as reflected by the accumulation of a specific breakdown product of the cytoskeletal protein, spectrin [2,28,30,34]. It is therefore likely that NR2 subunits would be truncated under the same experimental conditions. Results obtained with kainate treatment of hippocampal slices in culture indicated that kainate-induced calpain activation does result in NR2 subunit truncation. Possibly, calpain-mediated modifications of NMDA receptors provide for a long-lasting feed-back mechanism directed at limiting the consequences of NMDA receptor activation.

Acknowledgements

This work was supported by grants from NINDS NS18427 and Sankyo.

References


