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Specific proteolysis of the NR2 subunit at multiple sites by calpain

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Abstract

The NMDA subtype of glutamate receptor plays an important role in the molecular mechanisms of learning, memory and excitotoxicity. NMDA receptors are highly permeable to calcium, which can lead to the activation of the calcium-dependent protease, calpain. In the present study, the ability of calpain to modulate NMDA receptor function through direct proteolytic digestion of the individual NMDA receptor subunits was examined. HEK293t cells were cotransfected with the NR1a/2A, NR1a/2B or NR1a/2C receptor combinations. Cellular homogenates of these receptor combinations were prepared and digested by purified calpain I *in vitro*. All three NR2 subunits could be proteolyzed by calpain I while no actin or NR1a cleavage was observed. Based on immunoblot analysis, calpain cleavage of NR2A, NR2B and NR2C subunits was limited to their C-terminal region. *In vitro* calpain digestion of fusion protein constructs containing the C-terminal region of NR2A yielded two cleavage sites at amino acids 1279 and 1330. Although it has been suggested that calpain cleavage of the NMDA receptor may act as a negative feedback mechanism, the current findings demonstrated that calpain cleavage did not alter [^{125}I]MK801 binding and that receptors truncated to the identified cleavage sites had peak intracellular calcium levels, ^{45}Ca uptake rates and basal electrophysiological properties similar to wild type.

Keywords: calpain, excitotoxicity, long-term potentiation, NMDA receptor, proteolysis.

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NMDA receptors are ionotropic glutamate receptors that play important roles in learning and memory as well as numerous neurological disorders (Choi 1988; Collingridge and Lester 1989; Meldrum and Garthwaite 1990). These receptors exist as heteromultimers composed of two types of subunits denoted as NR1 and NR2. The NR1 subunit family consists of eight splice variants (NR1a–h) while the NR2 family is composed of four members (NR2A–D), each made from a separate gene (Nakanishi 1992). Functional NMDA receptors require members from each family and, *in vivo*, probably exist as a tetramer or a pentamer (Monyer *et al*. 1992; Monyer *et al*. 1994; Lynch *et al*. 1995; Hawkins *et al*. 1999).

Each subunit of the NMDA receptor has an extracellular N-terminus, three transmembrane domains and one intramembrane loop. The C-terminus of all subunits is intracellular, and may link the receptor to intracellular signaling pathway systems (Niethammer *et al*. 1996) such as neuronal nitric oxide synthase (Dawson and Dawson 1998), protein kinase C (PKC) (Fukunaga *et al*. 1992), p21^{ras} (Yun *et al*. 1998), phosphatidylinositol 3-kinase, and immediate early gene expression (Shan *et al*. 1997). Interactions of the receptor with signal transduction pathways can be modulated by the association of the NMDA receptor C-terminal tail with anchoring proteins and other cytoskeletal elements (Bi *et al*. 1998a; Wechsler and Teichberg 1998; Caputi *et al*. 1999; Sattler *et al*. 1999; Takagi *et al*. 2000) and are NMDA receptor subtype specific. For example, yotiao and PKC modulate NMDA receptors in subunit-specific manners based on the presence or absence of certain C-terminal regions (Grant *et al*. 1998; Lin *et al*. 1998; Westphal *et al*. 1999). The importance of the C-terminal region in proper NMDA receptor function is further exemplified by the findings of Sprengel *et al*. (1998), which demonstrated that gene-targeted mice lacking the C-terminal tail of the NR2 subunit exhibited phenotypic properties similar to mice with

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Abbreviations used: HBSS, HEPES-buffered saline solution; MEM, minimal essential medium, MK801, dizocilpine; PBS, phosphate-buffered saline; PKC, protein kinase C; SDS, sodium dodecyl sulfate.

a complete absence of an NR2 subunit. This occurred even though receptors lacking the C-terminal region of NR2A or 2C were electrophysiologically similar to wild-type receptors (Sprengel *et al*. 1998). This finding strongly suggests that post-translational or activity-dependent processing of the C-terminal tail could play a critical role in the modulation of NMDA receptor activity and/or function.

One means of post-translational modification of the C-terminus is proteolytic processing by calpain. Calpain, most commonly activated by calcium entry through NMDA receptors (Bahr *et al*. 1995; Adamec *et al*. 1998; Hewitt *et al*. 1998; Minger *et al*. 1998), regulates numerous enzymes and membrane-associated proteins including cytoskeletal components, integral membrane proteins and receptors (Johnson and Guttmann 1997). Calpain activity is inhibited by calpastatin, the only known endogenous and selective inhibitor of calpain (Johnson and Guttmann 1997). Recently, Bi *et al*. have shown that the NR2A and to a lesser degree the NR2B subunit are substrates of calpain *in vitro* (Bi *et al*. 1998a,b, 2000) and a single cleavage site in NR2A was identified. Although not directly tested, the authors hypothesized that calpain cleavage may represent part of a negative feedback mechanism to down-regulate NMDA receptor activity. The present study demonstrates that calpain I readily proteolyzes all three of the major NR2 subunits and that at least two cleavage sites exist for all NR2 subunits. In addition, the current study shows that calpain cleavage of NR2A-containing receptors does not alter basic NMDA receptor properties including calcium uptake, [^{125}I]MK801 binding or electrophysiological measurement.

Materials and methods

Materials

Glutamate, glycine, ketamine, aprotinin, pepstatin, phenylmethylsulfonyl fluoride and anti-actin were from Sigma (St Louis, MO, USA); minimal essential medium (MEM) serum, penicillin/streptomycin and glutamine were from Gibco (Rockville, MD, USA). Fetal bovine serum was from Hyclone (Logan, UT, USA); Dizocilpine (MK801) was from Research Biochemicals Inc. (Natick, MA, USA), [^{125}I]MK801 and $^{45}CaCl_2$ were from NEN (Boston, MA, USA) and HEK293t cells were from ATCC (Manassas, VA, USA; with permission of Dr Michelle Calos). Antibodies to the C-terminal portions of NR2A/2B (AB1548) and NR1a (AB1516), with epitopes at amino acids 1445–1464 and 909–938, respectively, were from Chemicon (Temecula, CA, USA). AB1548 recognized single prominent bands of either NR2A or NR2B and AB1516 recognized two bands of NR1a [due to differential glycosylation (Chazot *et al*. 1992)] by western blot of transfected cells. An N-terminal antibody (amino acids 25–130 of NR2C) which cross-reacts with NR2A and NR2B (A-6475) was from Molecular Probes (Eugene, OR, USA), which recognized single bands of either NR2A or NR2B with two bands of NR2C [possibly due to glycosylation (Chazot *et al*. 1992)] observed in our transfected cell model. Porcine calpain I was purchased from Calbiochem (San Diego, CA, USA). Rabbit calpastatin cDNA was a generous gift from Dr Masatoshi Maki. NR2A and NR2C cDNAs were from rat while NR2B was from mouse (Lynch *et al*. 1995; Gallagher *et al*. 1996).

Methods

Transfection of HEK293t cells

HEK293t cells were grown on tissue culture grade dishes (Corning Brand) in MEM media containing 5% horse serum, 5% fetal bovine serum supplemented with 2 mM glutamine, 100 units/mL penicillin/streptomycin and placed in a 5% CO_2 incubator at 37°C. Transfection of HEK293t cells with cDNA was accomplished by the method of Chen and Okayama (1988). Twenty-four hours following transfection, the media was changed and treatments were added. Ketamine (500 μM) was added to the media during transfection to prevent NMDA receptor activation as previously described (Grant *et al*. 1997). Using HEK293t cells, the transfection efficiency was approximately 70%.

Analysis of NMDA receptor subunit degradation by purified calpain I

Twenty-four hours after transfection, cells were rinsed with phosphate-buffered saline (PBS) and then scraped into 40 mM HEPES (pH 7.2) containing 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 10 μg/mL pepstatin. Samples were briefly sonicated on ice and stored in aliquots at −80°C until use. For *in vitro* proteolysis, the homogenates were incubated in the presence of varying concentrations of calpain I (0.25–2.5 units/mL) for up to 30 min in a buffer containing final concentrations of 1 mM $CaCl_2$, 5 mM dithiothreitol, and 40 mM HEPES, pH 7.2.

Development of fusion protein and shortened NR2A constructs

For fusion proteins, portions of cDNAs encoding C-terminal regions of NR2A and N-terminal regions of NR1a, 2A and 2C were excised using restriction enzymes and subcloned into pRSET vectors (Invitrogen, Carlsbad, CA, USA) carrying the sequence for a six-histidine tag in the N-terminus. The 5′ or 3′ terminus of the receptor portion was modified using PCR cloning to facilitate cloning into the pRSET expression system in the correct reading frame. The fusion proteins utilized in this study were constructed as follows: FP2A1464 contains bases 3348–4395 inserted as a *Bgl*II fragment (Gallagher *et al*. 1997); FP2A1400 contains bases 3348–4200 and was made by PCR by priming NR2A in *prk7* with the forward oligonucleotide 2AXHO5′ (GAGAAATCGGACCCTCGA GCTAAAGAGTCCTAGGTAT) and the reverse oligo 3′Stop4200 (ATGACCGAAGGAATTCTCAATAGCTGTCATTTACCGCCT). The sample was then digested with *Bgl*II and *Eco*RI and ligated into *prk7* and then subcloned into pRSET. FP2A1300 was produced by a similar strategy except that the 3′ oligonucleotide introduced a stop codon at amino acid 1300 (3′stop3900 = AGATAGAGT GAATTCTCATCTGGGTTTGTCCAGAATGTT); FP1ANT contains bases 804–1014 inserted as an *Eco*R1 fragment where the 5′ end was produced by PCR introduction of an EcoR1 site at base 804; FP2AENT contains bases 414–795 inserted as a *Bam*H1/*Sac*I fragment; FP2CNT contains bases 438–795 inserted as a *Pst*I fragment. These proteins were made by overexpression in *Escherichia coli* strain BL21 LYS, which carries the T7 RNA polymerase in lysogenic form, using induction with IPTG. The six-histidine

tagged fusion proteins were purified by nickel affinity chromatography with elution by low pH.

The NR2A1051 shortened form was constructed as previously described (Grant *et al*. 1998). This construct maintains the epitope tag required for immunoreactivity to antibody AB1548 consisting of the last six amino acids of the NR2A subunit.

In vitro *proteolysis of fusion proteins by calpain and sample preparation for Edman degradation*

Purified fusion proteins (0.1 mg/mL) were incubated with calpain I (0.05 units/mL) in a buffer containing 40 mM HEPES (pH 7.2), 5 mM dithiothreitol and 1 mM $CaCl_2$, at 37°C. Reactions were initiated by addition of calcium. Aliquots were removed at various time points, added to an equal volume of 2 × Laemmli stop buffer, then heated in a boiling water bath and stored at −20°C until use. In cleavage site determination studies, samples were similarly prepared except the reaction was stopped by addition of excess EGTA and the samples concentrated by SpeedVac (Brinkman Instruments, Westbury, NT, USA). Samples were resuspended in 1X Laemmli stop buffer and separated using a Tris-Tricine gel as described (Schagger and von Jagow 1987) except that 0.04% thioglycolic acid was added to the running buffer. Following transfer in 10% methanol buffered with 10 mM CAPS pH 11.0 to Problott (Applied Biosystems, Foster City, CA, USA), the blot was stained with Coomassie brilliant blue and the appropriate bands excised and submitted for protein sequencing. Protein sequencing was provided by the Protein Chemistry Laboratory of the School of Medicine of the University of Pennsylvania (supported by core grants of the Diabetes and Cancer Centers DK-19525 and CA-16520).

Western blotting

For HEK293t cellular homogenates, 30 μg of total protein was loaded on a 7% polyacrylamide gel. Following sodium dodecyl sulfate (SDS) gel electrophoresis, proteins were transferred to nitrocellulose, blocked with 5% non-fat dry milk, incubated with primary antibodies to NR2A, NR1a or actin. Blots were then incubated with appropriate HRP-conjugated secondary antibodies and developed with enhanced chemiluminescence (Pierce Chemical Co., Rockford, IL, USA). For six-histidine tagged fusion protein constructs, samples were similarly treated except that a 12% polyacrylamide gel was used and following transfer to nitrocellulose, the proteins were stained using the India HisProbe (Pierce Chemical Co., Rockford, IL, USA) and visualized with enhanced chemiluminescence. Each blot was quantitated using imaging densitometry and analyzed using NIH IMAGE software. Statistical differences were determined by ANOVA and considered significant at $p < 0.05$.

Calcium uptake assay

Twenty-four hours after transfection, six-well plates of HEK-293t cells were washed twice with HEPES-buffered saline solution (HBSS; 140 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 2.6 mM dextrose, 20 mM HEPES, pH 7.5) without $CaCl_2$ and 2×10^6 cpm of ^{45}Ca in HBSS was added to each well. Glutamate (100 μM) and 100 μM glycine in the presence or absence of 100 μM MK801 was then added to activate or inhibit NMDA receptor activity. Plates were incubated at room temperature (22–24°C) for 10 min and then washed once with HBSS containing 2 mM $CaCl_2$. Cells were then harvested by addition of 500 μL 0.05% trypsin for 5 min. Twenty milliliters of scintillation cocktail was then added and the radioactivity quantified using a Beckman (model LS 5000TD) scintillation counter. Triplicate well determinants were averaged in each independent experiment. Statistical differences were determined by two sample unpaired *t*-test.

Measurement of intracellular calcium concentrations

Twenty-four hours after transfection, cells were washed once with HBSS, then loaded with 1 μM fura2-AM for 30 min at 37°C. fura2-loaded cells were washed once with HBSS and then placed onto the stage of an inverted Nikon Eclipse TE300 microscope. Intracellular calcium changes were determined based upon the calibration curve of Grynkiewicz: $[Ca^{2+}] = K_D b[(R - R_{min})/(R_{max} - R)]$ (Grynkiewicz *et al*. 1985). For the present study the calibrated values used were $R_{min} = 0.22$, $R_{max} = 5.0$, and $b = 6.3$. The K_D was assumed to be 135 nM. Transfected cells were treated with 100 μM glutamate and 100 μM glycine for 60 s. Data were acquired and calculated using Metafluor software (Universal Imaging, West Chester, PA, USA). Peak intracellular calcium levels were typically observed in less than 10 s.

[^{125}I]MK801 binding

Cell membranes were prepared as previously described using preparations which remove MK801 and other NMDA receptor antagonists (Williams *et al*. 1991; Lynch *et al*. 1995; Gallagher *et al*. 1996). Membranes were incubated in saturating glycine (100 μM) and glutamate (100 μM) with 300 pM [^{125}I]MK801 (Lynch *et al*. 1994). Typical assays of NR1a/2 A and NR1a/2B yielded binding levels of approximately 30–40 fmol/mg protein at this [^{125}I]MK801 concentration with total to non-specific ratios were greater than 4 : 1. Membranes were harvested (Brandel Harvester) onto PEI coated glass fiber filters (Schleicher and Schuell, Keene, NH, USA), and the radioactivity quantified using a Beckman (model 5500B) gamma counter. Using this protocol, binding to NR1a/2A combinations was observed while no binding to either NR1a or NR2A alone was detected (Lynch *et al*. 1994). For inhibition curves, MK801 was added to assay tubes at concentrations ranging from 0.1 nM to 10 μM. Data were analyzed as inhibition curves using software derived from the NIH Prophet system, and IC_{50} values were determined. Data were converted to log IC_{50} values for statistical analysis. Statistical differences at $p < 0.05$ were determined by two-sample unpaired *t*-test.

Electrophysiological measurements

Whole-cell voltage-clamp recordings were conducted at room temperature on a Leica (Exton, PA, USA) inverted microscope equipped with Hoffman modulation contrast optics. Transfected HEK cells were voltage clamped at − 70 mV and signals were recorded and amplified with an Axopatch 1D (Axon Instruments, Foster City, CA), and filtered at 5 kHz. Data acquisition and analysis was performed with a digidata 1200 A/D converter and pClamp 6.0 software (Axon Instruments, Union City, CA, USA). Electrodes were fabricated from thick wall borosilicate glass (World Precision Instruments, Sarasota, FL, USA) and pulled to a resistance of 2–6 MΩ when filled with an internal solution composed of: 135 mM CsCl, 2 mM $MgCl_2$, 4 mM MgATP in 10 mM HEPES (pH 7.25) on a two-stage puller. The external bath solution consisted of 155 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. A junction potential of 0.95 mV was

measured between the internal and external solutions. All data were left uncorrected for this junction potential. Current voltage relationships were calculated from the response to ramp clamp currents by incrementing the voltage from −90 to + 80 mV over a 1-s duration. Voltage ramps were performed in control and glutamate (100 μM) and glycine (100 μM) containing external solution applied via a large bore multibarrel concentration clamp apparatus (200 μm internal barrel diameter). Control ramps were then subsequently subtracted from experimental ramps to provide leak-subtracted NMDA receptor activated currents. Two-tailed unpaired Student's *t*-tests were performed to determine statistical significance at the $p < 0.05$ confidence level when comparing different treatment groups in the electrophysiological experiments.

Results

In vitro proteolysis of NR subunits by calpain

To assess whether the major NMDA receptor subunits were substrates for calpain, the ability of purified calpain to proteolyze NR1 or NR2 subunits in HEK 293t cell homogenates was examined. While NR2A was readily digested by calpain I, no significant decrease in either NR1a or actin immunoreactivity was observed (Fig. 1a). All three of the major NR2 subunits were calpain substrates with similar rates of digestion (Fig. 1b). In addition to a significant decrease in the amount of substrate, multiple stable breakdown products were observed when the samples were probed with an N-terminal antibody: molecular weights of approximately 140 and 130 kDa for NR2A (NR2A contains 1464 amino acids); 140, 130 and 120 kDa for NR2B (NR2B contains 1483 amino acids); and 110 and 100 kDa for NR2C (NR2C contains 1238 amino acids) (Fig. 2a). Co-transfection of NMDA receptors with calpastatin blocked the formation of these products as illustrated with the lack of NR2A digestion in calpastatin versus control vector transfected cells in Fig. 2(a). In the case of NR2A, fragments of molecular weights 20 and 15 kDa were observed with a C-terminal directed antibody (Fig. 2b). Identical patterns of stable NR2 subunit breakdown products were observed regardless of the amount of calpain studied from 0.05 to 2.5 units/mL.

Based on the molecular weight migration of the stable breakdown products observed with either the N-terminal and C-terminal antibodies, it appeared that calpain cleavage of the NR2 subunits occurred in the C-terminal region near amino acid 1300. To further demonstrate the location and selectivity of the *in vitro* digestion of NR2 subunits, an NR2A subunit was constructed that was truncated at amino acid 1051 which also contained the final six amino acids required for detection by immunoblot (AB1548). This construct was not degraded by calpain I *in vitro*, suggesting that calpain cleavage sites had been removed by this truncation (Fig. 2c).

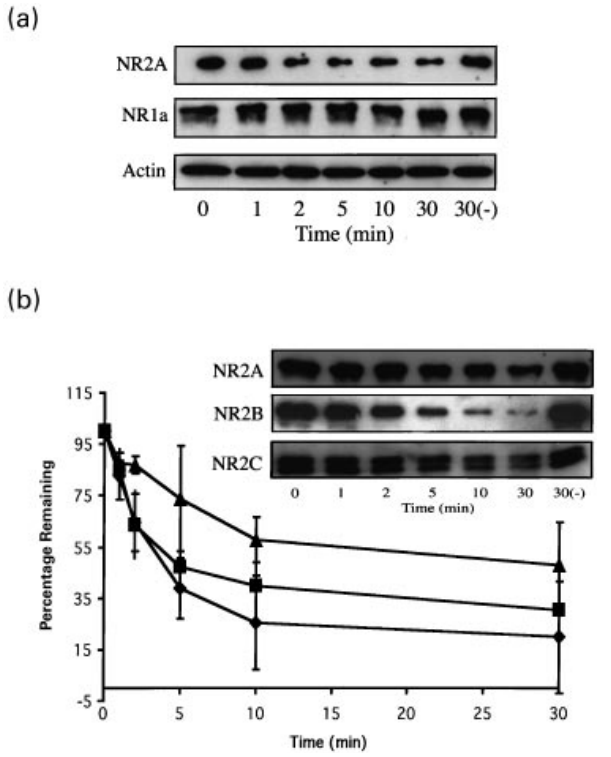

Fig. 1 *In vitro* proteolysis of NR2A, NR2B and NR2C by calpain I. Representative immunoblots (a) of NR2A, NR1a and actin digested with purified calpain I (0.1 units/mL) for up to 30 min. While NR2A was rapidly degraded, neither NR1a nor actin was significantly proteolyzed. All three NR2 subunits were readily degraded by calpain with a 50–70% decrease in immunoreactivity at 30 min while no degradation was observed in the absence (–) of calcium (inset, b). Quantitated data ± SEM of NR2 subunit degradation (NR2A, ◆ NR2B, ■; NR2C, ▲) by calpain (0.6 units/mL) *in vitro* (b) are expressed in terms of percent of initial substrate versus time ($n = 3$). NR2A and NR2B were probed with antibody AB1548 while NR2C was probed with antibody A-6475).

In vitro proteolysis of NMDA receptor fusion protein constructs

In order to identify the location of the cleavage sites within NR2A, fusion protein constructs were made containing various portions of the intracellular C-terminal region of the NR2A subunit. Control fusion proteins composed of the N-terminal portion of various NMDA receptors were also generated. The regions of the C-terminal tail of NR2A were selected based upon the approximate locations of calpain cleavage sites as determined from *in vitro* digests of the full length NR2A (see Figs 1 and 2). The amino acid sequence range, and calculated molecular weights of the NMDA receptor subunit present in each fusion protein are indicated in Table 1. Several of the fusion protein constructs migrated slightly larger than these calculated masses as detected on SDS–polyacrylamide gels (Fig. 3). The various fusion protein constructs were incubated with purified calpain I and

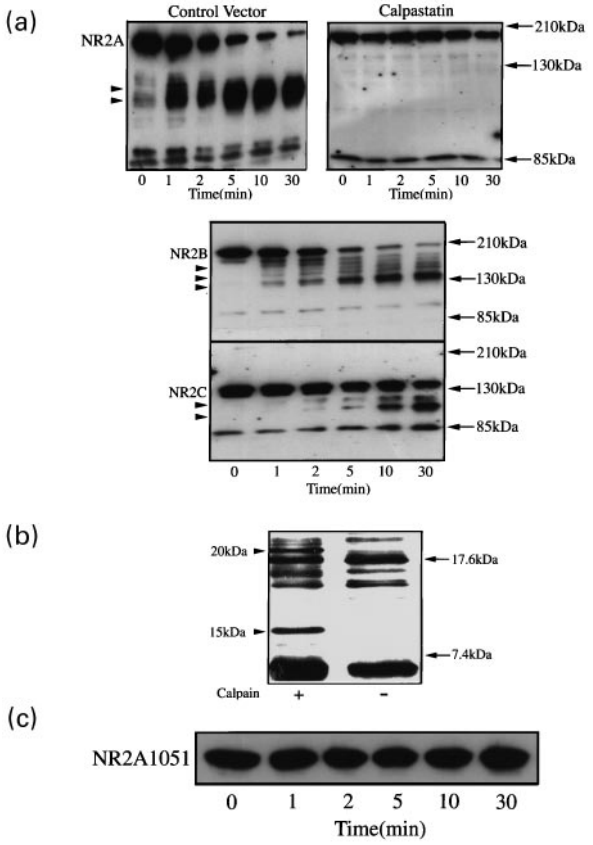

Fig. 2 Identification of stable N- and C-terminal fragments generated by calpain proteolysis. Representative immunoblots demonstrating the formation and stability of N-terminal fragments (using antibody A-6475) of all three NR2 subunits (a) and inhibition of calpain digestion of NR2A by cotransfection with calpastatin (a, compare control vector versus calpastatin) during *in vitro* digestion by calpain I from three to five separate experiments. Stable N-terminal fragments of NR2A and NR2B were readily formed in as little as 1 min, which remained stable to further calpain proteolysis while the intact subunit was continually degraded. NR2C fragments were slower to form and reach detectable levels but remained stable over time following their creation. In the case of NR2C (see Fig. 2a, bottom blot), this gel was overexposed to highlight the formation of the N-terminal fragments and thus a decrease in immunoreactivity of intact NR2C is not observed. In addition, overexposure partially masks the presence of the intact NR2C doublet, which may result from the presence or absence of glycosylation (Chazot *et al.* 1992) as observed in Fig. 1. (b) is a representative immunoblot identifying two C-terminal fragments generated from the 30-min digestion of NR2A by calpain (+) compared with the incubation of NR2A in the absence of calpain addition (−). (c) is a representative immunoblot of a time course digestion of NR2A1051 by calpain I. No digestion of this construct was observed for up to 30 min indicating that no calpain sites are present within this construct. Arrowheads highlight the approximate locations of breakdown product formation for each subunit and the approximate location of molecular weight standards are indicated to the right of each blot.

Table 1 Amino acid composition range of fusion protein constructs

Fusion protein	Amino acid range	Molecular weight (kDa)
FP1aNT	268–338	11 (13)
FP2AENT	138–265	18 (24)
FP2CNT	146–265	17 (28)
FP2A1464	1116–1464	45 (48)
FP2A1400	1116–1400	37 (42)
FP2A1300	1116–1300	26 (32)

The range of amino acids for each fusion protein as well as the calculated molecular weights are listed. Also indicated in parentheses is the apparent molecular weight as observed on SDS-polyacrylamide gels. Numbering is based upon the respective rat subunit. Each fusion protein also contained 40 amino acids of leader sequence from the pRSET vector.

examined by immunoblot using the India HisProbe, which recognizes the six-histidine tag contained in the N-terminal portion of every fusion protein constructed. Fusion protein FP2A1400 was evaluated initially, and the rate and extent of calpain-mediated proteolysis was studied (Fig. 3a). FP2A1400 was readily degraded by calpain with a 50% decrease in detection by India HisProbe reagent after 2 min, and was almost completely digested by 5 min. Two stable breakdown products that migrated on SDS–polyacrylamide gels at molecular weights of approximately 40 and 30 kDa were easily detectable after 1 min, and remained present throughout the 30-min time course (see Fig. 3a, inset). N-terminal constructs of NR1a, NR2A and NR2C were not degraded (Fig. 3b), showing that calpain cleavage is selective for the C-terminal region and that no cleavage sites exist within the extreme N-terminus of the fusion protein containing the six-histidine tag. These results suggested the presence of two selective sites for cleavage of the NR2A subunit by calpain in the final 200 amino acids.

Identification of calpain-mediated cleavage sites within NR2A

The other fusion protein constructs derived from the C-terminus of NR2A were used to narrow the search for these two cleavage sites. Proteolysis of these additional proteins (Fig. 4a) suggested two conserved cleavages (see Arrows in Fig. 3a) flanking amino acid 1300. Due to the relative insolubility of FP2A1464 (the longest fusion protein) and the fact that both identifiable cleavages were within FP2A1400 based upon immunoblotting analysis, FP2A1400 was used for cleavage site identification by Edman degradation. To determine the two cleavage sites that were preliminarily identified by comparative immunoblotting of the various 2A fusion proteins, FP2A1400 was cleaved *in vitro* by calpain I and the C-terminal fragments separated

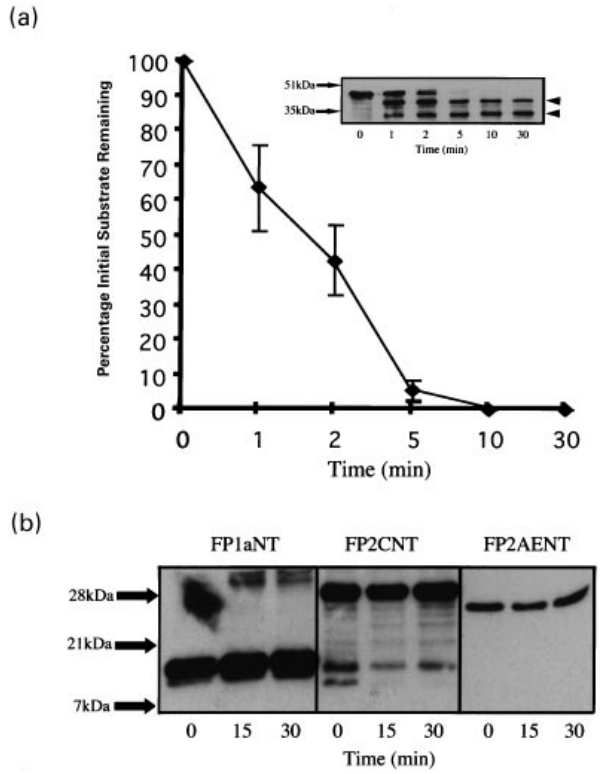

Fig. 3 Determination of the selective proteolysis of the C-terminus of NR2A *in vitro* by fusion protein digestion. Quantitated data ± SEM are expressed in terms of percent initial substrate remaining. FP2A1400 was rapidly degraded by calpain with 50% of the initial substrate degraded within 2 min and nearly completely digested within 10 min (a). A representative immunoblot of FP2A1400 degraded *in vitro* with calpain I is also shown (see inset). Two stable breakdown products were formed as detected by the N-terminal stain India HisProbe (see arrowheads). N-terminal fusion protein constructs FP1aNT (11 kDa), FP2CNT (17 kDa) and FP2AENT (18 kDa) were not degraded by calpain I *in vitro* (b). Arrows indicate migrations of molecular weight markers.

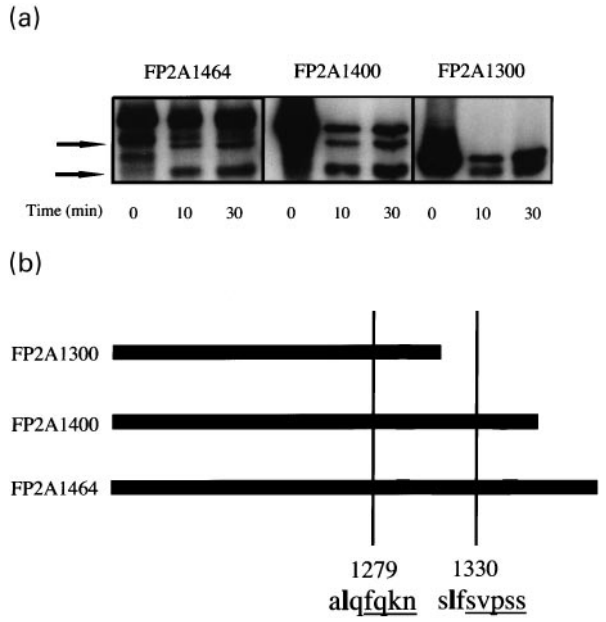

Fig. 4 Identification of calpain-mediated cleavage sites by fusion protein digestion. (a) shows representative India HisProbe stained blots of three fusion protein constructs containing differing C-terminal portions of NR2A degraded *in vitro* by calpain I for 10 and 30 min. Blots shown in A were overexposed to more readily observe the breakdown products of each fusion protein and thus are not within the linear range of detection. The top bands in all lanes represent intact, undigested fusion protein. All fusion proteins generated breakdown products of the same molecular weights (see arrows), consistent with the presence of specific conserved calpain-mediated cleavage sites. A diagrammatic representation of each fusion protein is shown in (b). The calpain-mediated cleavage sites identified by Edman degradation from FP2A1400 are indicated by vertical lines and the amino acid number is shown (numbering is based upon rat NR2A sequence). The amino acids surrounding the cleavage site are indicated and the first four amino acids sequenced by Edman degradation are underlined. Although no consensus sequence is known for calpain, there is a prototypical leucine located in the P2′ position (indicated in bold).

by Tris-Tricine polyacrylamide gel electrophoresis. Following transfer and total protein staining, the bands of interest were excised and submitted for sequencing by Edman degradation. Based upon the amino acid sequence obtained (underlined sequence), the two cleavage sites were identified at amino acids 1279 and 1330 (Fig. 4b). Although no primary amino acid consensus sequence is known for calpain cleavage, these sites correspond to a common theme of calpain cleavage with a leucine in the P2′ position (Murachi 1989). Cleavage at these putative sites would produce C-terminal fragments with molecular weights of approximately 15 and 20 kDa in agreement with the molecular weights observed on immunoblots of the digests of full-length NR2A *in vitro* (see Fig. 2).

Effects of truncation at calpain cleavage sites within NR2A on [^{125}I]MK801 binding, calcium imaging, ^{45}Ca uptake and electrophysiological properties

To determine whether calpain proteolysis alters NMDA receptor function, the effects of calpain proteolysis on NMDA receptor mediated calcium influx and [^{125}I]MK801 binding were studied. [^{125}I]MK801 binding serves as a marker of intact receptors, as no significant binding of [^{125}I]MK801 is observed in cells transfected with individual subunits alone (e.g. NR1a or NR2A) (Lynch *et al*. 1994, 1995). Similarly, the calcium uptake assay and electrophysiology serve as markers of physiologically active receptors, as channel activity is only present when a receptor contains members of both families of NMDA

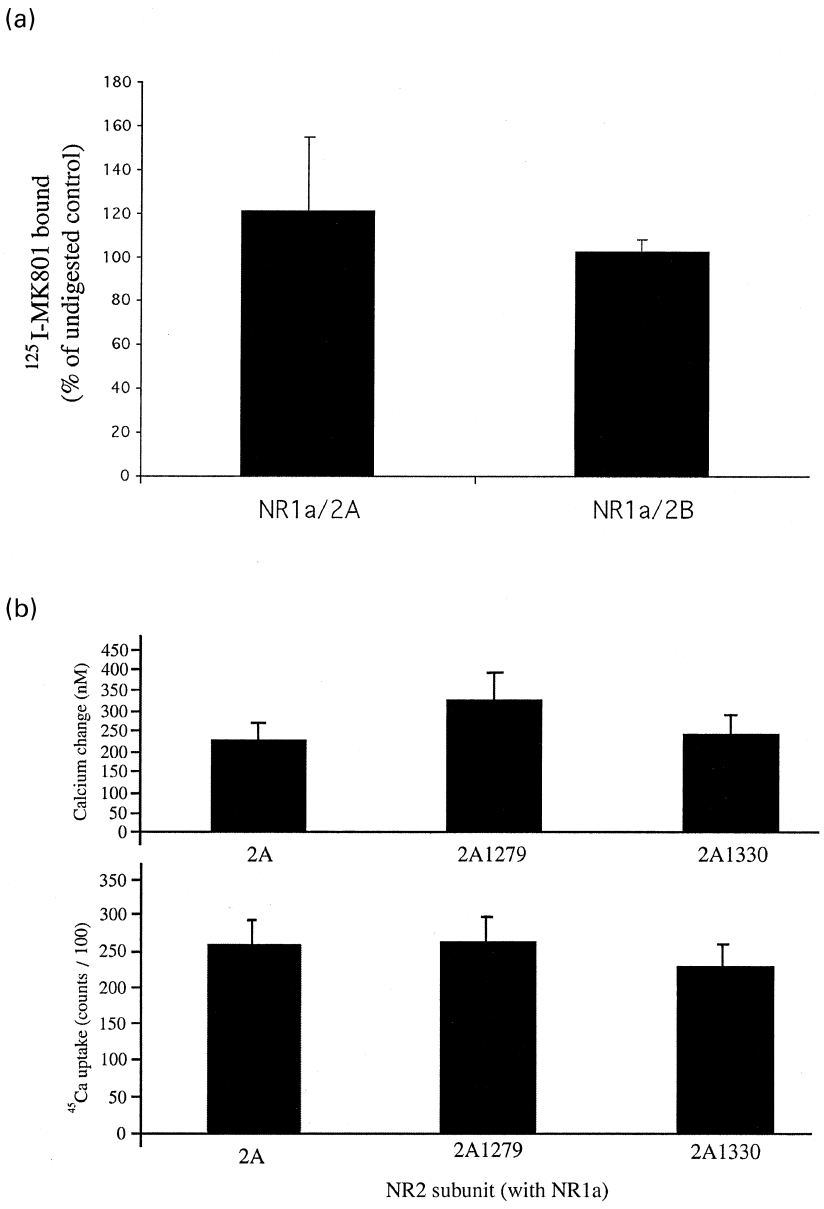

Fig. 5 Determination of effects of calpain cleavage on NMDA receptor activity measurements. Quantitated data ± SEM of [^{125}I]MK801 binding of calpain treated NR1a/2A or NR1a/2B receptor combinations is shown in (a). HEK cells were transfected with either NR1a/2A or NR1A/2B, membranes were harvested and each transfection was separated into two samples. One of these samples was digested with calpain for 30 min while the other was treated with buffer alone. Membranes were then assessed for the ability to bind [^{125}I]MK801. Data are shown as percentage binding in calpain treated membranes compared with untreated control and are mean ± SEM of 3–5 experiments. Calpain treatment had no effect on the ability of receptors of either NR1A/2A or NR1a/2B to bind [^{125}I]MK801. Constructs of NR2A shortened to the *in vitro* identified calpain cleavage sites of amino acids 1279 (NR2A1279) or 1330 (NR2A1330) by PCR were cotransfected with NR1a (see Methods) and the ability of these combinations to increase intracellular calcium levels and increase extracellular calcium uptake were compared with wild type (b). No statistical difference was observed in either ^{45}Ca uptake ($n = 6$) or peak intracellular calcium change ($n = 4$–6) measurement paradigm although the construct shortened to 1279 was elevated compared with wild-type NR2A.

receptor subunits (i.e. combinations of both NR1a-h and NR2A-D) (Grant *et al.* 1997). Thus, measurement by these two methods can determine whether the effects of calpain proteolysis can have functional consequences.

To determine whether the truncated forms of NR2A or NR2B remained associated with NR1a and maintained activity, membranes from cells transfected with NR1a/2A or NR1a/2B were treated with calpain I *in vitro* as shown in Fig. 1(b), and [^{125}I]MK801 binding was assessed. No differences were noted in the level of [^{125}I]MK801 binding between the calpain digested samples and undigested controls (Fig. 5a). This suggested that cleavage of the C-terminal region of either NR2A or NR2B by calpain does not alter the NR1a/NR2 combined receptor's binding characteristics. Similarly, receptors made from NR2A subunits truncated to the exact site of calpain proteolysis were assayed for MK801 affinity using ligand binding. No statistically significant differences were observed in the affinity of MK801 for receptors created by NR1a/2A, NR1a/2A1051, NR1a/2A1330 or NR1a/2A1279 ($n = 5$–7, paired t-test, $p > 0.05$, data not shown). This further suggested that proteolysis by calpain does not alter the affinity of NMDA receptors for MK801 following calpain cleavage.

NR2A1279 and NR2A1330 shortened constructs cotransfected with NR1a were also assayed for their ability to increase intracellular calcium levels or conduct calcium from the extracellular space compared with NR1a/2A wild type. No significant difference in either intracellular calcium

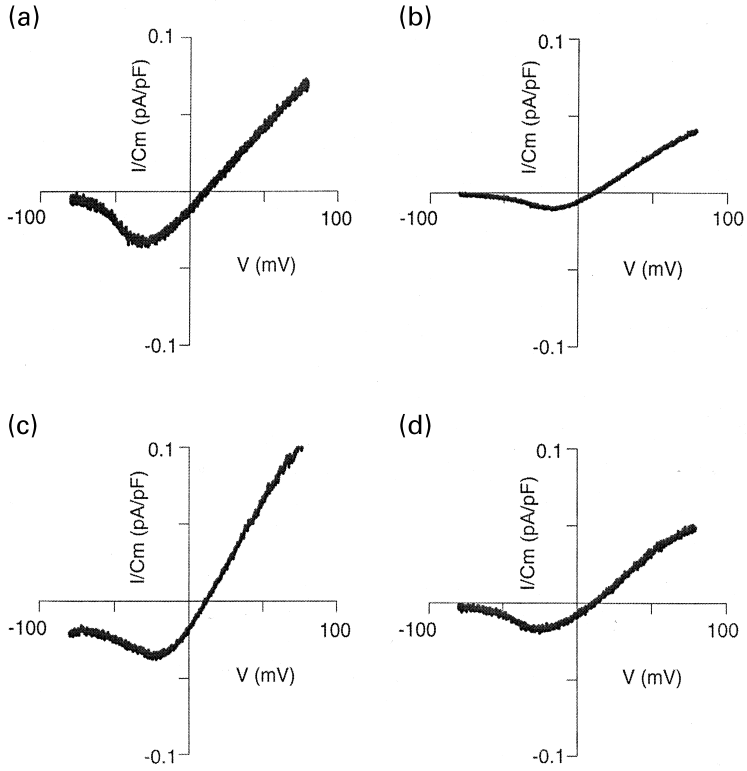

Fig. 6 Capacitance normalized current voltage (*I–V*) relationships for wild type (1a/2 A) (a), NR2A1051 (b), 2A1279 (c) and 2A1330 (d) generated in response to a ramped voltage change from −90 to +80 mV (see methods for details). The curves, leak subtracted, are recordings generated in glutamate and glycine (100 μM each) subtracted from the identical voltage protocol in control solution.

change (approximately 250 nM) or ^{45}Ca uptake was observed between these shortened constructs and wild-type receptor following agonist stimulation (Fig. 5b).

These same constructs were further assessed using electrophysiological techniques in HEK 293t cells. In order to more fully assess the putative physiological changes in the shortened constructs, the current–voltage (*I–V*) relationships generated by a depolarizing ramp voltage protocol (see methods) present in the four experimental groups were examined. All *I–V* curves had a characteristic N-shape with a negative slope region between −50 and −30 mV, and reversed at approximately + 15 mV. Furthermore, to determine whether the constructs had any significant physiological ramifications, four different measurements were compared. First, the voltage at which the inward current was greatest; second, the magnitude of that normalized current (calculated by dividing the peak inward current by cellular capacitance); third, the reversal potentials (E_{rev}); and, fourth, the normalized peak outward current (measured at +55 mV) in the four populations. The *I–V* curve for the wild-type (NR1a/2 A) construct had its peak inward current at −31.0 ± 3.4 mV (Fig. 6a, $n = 5$), and reversed at + 14.8 ± 2.9 mV. These values were similar and not significantly different (one-way ANOVA) to those for NR1a/2A1051 (Fig. 6b, $n = 7$), NR1a/2A1279 (Fig. 6c, $n = 4$), and NR1a/2A1330 (Fig. 6d, $n = 4$) where the values for peak inward currents occurred at −28.8 ± 4.3; −32.9 ± 9.6; −33.5 ± 9.9 mV, and $E_{reversus}$ were 16.5 ± 11.8; 15.4 ± 4.7; 13.2 ± 3.6 mV for each construct, respectively. The normalized peak inward currents in all four groups ranged from −0.025 to −0.047 pA/pF; whereas, the normalized peak outward current measured at + 55 mV ranged from 0.027 to 0.07 pA/pF.

Discussion

The present study demonstrates that the three major NR2 subunits are substrates for the calcium activated protease calpain at multiple specific sites within their C-terminus *in vitro*. In addition, large N-terminal fragments of all three NR2 subunits and smaller C-terminal fragments of NR2A are created by calpain digestion and remain stable to further calpain digestion over time. Truncation at the C-terminus by calpain I or assessment of NR2A receptors shortened to the *in vitro* identified cleavage sites did not reveal changes in basic receptor properties compared with combinations containing intact, full length receptor.

Using *in vitro* cleavage on brain membranes, it has been suggested that calpain may act on NR2A and to a lesser extent NR2B subunits, with a site of cleavage at amino acid 1279 of NR2A (Bi *et al.* 2000). Additionally, it was hypothesized that calpain cleavage of NR2 subunits was related to down-regulation of NMDA receptor activity (Bi *et al.* 1998a, 1998b, 2000). The present study, however, suggests that the effect of calpain on NMDA receptors is generated at a larger number of distinct sites on NR2A, 2B

and 2C, and that these cleavages do not influence the basic functional properties of the receptor. The present findings suggest that the function of calpain cleavage is not likely to be primarily destructive as NMDA receptors containing calpain-cleaved subunits are stable and maintain basic properties similar to wild-type, full-length NMDA receptors. Although cleavage of the C-terminal region by calpain does not necessitate complete dissociation of the fragments generated, given the locations of the cleavages in the C-terminal region of NR2A, it seems likely that calpain cleavage alters more complex aspects of NMDA receptor function, including localization, modulation by second messengers, or receptor turnover.

The potential modulatory role of calpain in NMDA receptor function is consistent with calpain effects on other membrane-associated proteins. Several synaptic proteins have been shown to be physiological substrates of calpain, including neuronal nitric oxide synthase, CAM kinase, and other calcium channels (Dosemeci and Reese 1995; Hell *et al*. 1996; Hajimohammadreza *et al*. 1997). Typically, calpain proteolysis modifies substrate activity, as has been demonstrated for several classes of proteins (Johnson and Guttmann 1997). For example, L-type calcium channels have increased calcium permeability following calpain cleavage (Hell *et al*. 1996), while ryanodine receptor channel activity is decreased by calpain proteolysis (Shevchenko *et al*. 1998). In the case of NR2A, it appears that their ability to function, at least basally, subsequent to cleavage by calpain is maintained as calcium uptake, [^{125}I]MK801 binding and several key electrophysiological measurements were unchanged in either calpain digested samples or receptors containing shortened NR2A constructs.

Although receptor channel properties were not directly altered, NMDA receptors are likely to become dissociated from cytoskeletal anchors such as PSD-95 which interact with this C-terminal region (Steigerwald *et al*. 2000) and alter receptor function *in vivo*. Thus, cleavage by calpain could produce intermediate NMDA receptors separated from these proteins, leading to mobile NMDA receptors. Alterations of NMDA receptor localization following NMDA receptor activation have previously been observed, and the present findings suggest a potential mechanism for this event (Allison *et al*. 1998).

In addition to cytoskeletal interactions, the specific cleavage sites demonstrated in the NR2A receptor would be predicted to alter selective interactions with other second messenger systems (Grant *et al*. 1998). Recently, Bi *et al*. (2000) identified one of the calpain cleavage sites at amino acid 1279 which has been confirmed here and previously (Guttmann *et al*. 1999) and suggested that this site may be regulated by tyrosine kinase activity (Bi *et al*. 2000). Similarly, a PKC consensus sequence (S/T-X-R/K) is present adjacent to the second site found in the present study at amino acid 1330 of NR2A. This site may be responsible for the inhibition of calpain cleavage observed by PKC phosphorylation of NMDA receptors noted recently (Bi *et al*. 1998b). In addition, the cleavage sites identified here flank the expected site of CAM kinase phosphorylation of the NR2A subunit (Gardoni *et al*. 1999).

Calpain cleavage may alter NMDA receptors by several other mechanisms. For example, calpain cleavage might alter the modulation of the NMDA receptor by protein kinases such as PKC, CaMKII, or tyrosine kinases (Zheng *et al*. 1998) which have been shown to be involved in model systems of LTP (Lovinger *et al*. 1986; Hu *et al*. 1987; Caputi *et al*. 1999). Calpain processing of the NMDA receptor may release the NMDA receptor from synaptic anchors such as spectrin or PSD-95 (Takagi *et al*. 2000) within the postsynaptic density resulting in altered synaptic function. Alternatively, the low molecular weight products generated by the selective calpain cleavage of the NR2A subunit may have specific roles in neuronal function and pathology, as similar calpain-generated fragments from troponin-C have been shown to have in cardiac disease (Murphy *et al*. 2000).

In conclusion, the present findings demonstrate that the NR2 subunits appear to be specific substrates of the calcium-dependent protease, calpain. These data further suggest that cleavage of the NR2A subunit does not appear to alter basic channel properties compared with wild-type NR2A-containing NMDA receptors. Finally, calpain proteolysis was shown to occur in a region that may have important physiological consequences *in vivo* relating to both physiological and pathological conditions.

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