

## Src-mediated Tyrosine Phosphorylation of NR2 Subunits of N-Methyl-D-Aspartate Receptors Protects from Calpain-mediated Truncation of Their C-terminal Domains\*

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**Src-mediated tyrosine phosphorylation of N-methyl-D-aspartate receptor subunits has been shown to modify the functional properties of N-methyl-D-aspartate receptors. Moreover, calpain-mediated truncation of N-methyl-D-aspartate receptor subunits has been found to alter the structure of the receptors. In the present study, we first used immunoprecipitation with a variety of antibodies against N-methyl-D-aspartate receptor subunits and anti-phosphotyrosine antibodies to show that tyrosine-phosphorylated subunits of N-methyl-D-aspartate receptor are protected against calpain-mediated truncation of their C-terminal domains. A GST fusion protein containing the C-terminal domain of NR2A was used to identify the calpain cutting sites in the C-terminal domain. One site was identified at residues 1278–1279, corresponding to one of the preferred calpain truncation sites. This site is adjacent to a consensus sequence for Src-mediated tyrosine phosphorylation, and Src-mediated tyrosine phosphorylation of the GST-NR2A C-terminal fusion protein also inhibited calpain-mediated truncation of the fusion protein. We propose that phosphorylation of NR2 subunits and the resulting inhibition of calpain-mediated truncation of their C-terminal domains provide for the stabilization of the N-methyl-D-aspartate receptors in postsynaptic structures.**

investigated the role of tyrosine phosphorylation of the NR2 subunits and shown that such phosphorylation is associated with increased function of the receptors. In particular, Src-mediated phosphorylation of tyrosine residues in the C-terminal domain of the subunits prevents a Zn<sup>2+</sup>-dependent inhibition of the NMDA receptor channel, thereby resulting in increased channel conductance (18). The physiological significance of tyrosine phosphorylation is not yet clear, although it has been shown that tyrosine phosphorylation increased markedly under physiological or pathological conditions (19, 20). In particular, tyrosine phosphorylation is increased following long term potentiation (LTP) induction in hippocampus (21, 22).

Another type of posttranslational modification of NMDA receptors consists of calpain-mediated truncation of the C-terminal domains of NR2 subunits. Calpain is a neutral, calcium-activated protease, which has been implicated in both physiological and pathological modifications of synaptic efficacy (23–25). Membrane treatment with purified calpain produced a decrease in the amount of the 170-kDa species of NR2 subunits and the formation of lower molecular weight fragments still recognized by antibodies against the C-terminal domain of NR2A/B subunits (26). Incubation of frozen-thawed brain sections in the presence of calcium also results in a decrease in the amount of the 170-kDa species of NR2A/B recognized by these antibodies, and this effect is completely blocked by calpain inhibitors. Finally, kainic acid treatment of cultured hippocampal slices is accompanied by a decrease in the amount of the 170-kDa species of NR2A/B and the formation of lower molecular weight fragments of the subunits (26). We postulated that such an effect could be involved in the regulation of the turnover of NMDA receptors or in the relocation of the receptors since the C-terminal domains of NMDA receptors are involved in the targeting/anchoring of the receptors in synaptic membranes through interactions with a family of PDZ-containing proteins (27–29).

Interactions between phosphorylation and calpain-mediated regulation of NMDA receptors have also been reported (30). Increasing the state of phosphorylation of NMDA receptor subunits by incubating tissue sections in the presence of phosphatase inhibitors was found to decrease the extent of calpain-mediated truncation of NR2A/B subunits of NMDA receptors. However, no information was available concerning which phosphorylation site(s) was responsible for this effect, and the possible functions of the interactions between phosphorylation and calpain-mediated regulation of NMDA receptors. In the present study, we used immunoprecipitation with antibodies against phosphotyrosine as well as antibodies against NR2A and NR2B subunits to determine whether tyrosine phosphorylation interferes with calpain-mediated truncation of NR2A and/or NR2B subunits. Furthermore, we used a GST fusion protein to iden-

N-Methyl-D-aspartate (NMDA)<sup>1</sup> receptors are heteromeric proteins composed of two families of subunits, NR1 and NR2. Whereas a single gene codes for NR1 subunits with at least 8 splice variants, 4 different genes encode NR2 subunits, NR2A–D (1–5). Although the exact stoichiometric composition or the number of different configurations of the NMDA receptors is not yet known, it is widely admitted that NMDA receptors comprise mixtures of NR1 and NR2 subunits (6, 7). NMDA receptors are post-translationally regulated by several phosphorylation reactions (8–10). Phosphorylation of serine and threonine residues mediated by calcium/calmodulin protein kinase II as well as protein kinase C has been demonstrated (11–14). More recently, several laboratories (10, 15–17) have

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<sup>1</sup> The abbreviations used are: NMDA, N-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; LTP, long term potentiation; GST, glutathione S-transferase.

tify potential cutting sites by calpain in the C-terminal domain of NR2A. The results clearly indicate that Src-mediated tyrosine phosphorylation protects against calpain-mediated truncation of the C-terminal domain of NR2A subunits of NMDA receptors and suggest possible functions for calpain-mediated truncation of the C-terminal domains of NMDA receptors.

#### EXPERIMENTAL PROCEDURES

**Preparation of Synaptic Membranes and Calpain Treatment**—Synaptic membranes were prepared from telencephalon of adult Harlan Sprague-Dawley rats (200–250 g) as described previously (31). After the final centrifugation/resuspension in 50 mM Tris acetate buffer (pH 7.4) containing 0.1 mM EGTA, aliquots of synaptic membrane fractions were stored at  $-70^{\circ}\text{C}$  until use. Synaptic membranes ( $\sim 300\ \mu\text{g}$  of protein) were incubated at  $35^{\circ}\text{C}$  for 30 min in the presence or absence of purified calpain I (16 units/ml; Calbiochem) with or without 2 mM calcium (32). The reaction was terminated by centrifuging samples at  $4^{\circ}\text{C}$  for 15 min at  $25,000 \times g$ . Pellets were lysed in phosphate-buffered saline containing protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{ml}$  pepstatin A, 1  $\mu\text{g}/\text{ml}$  leupeptin) and 2 mM EDTA. Membranes were then sonicated and centrifuged at  $4^{\circ}\text{C}$  for 10 min at  $100,000 \times g$ . Synaptic membranes were resuspended in lysis buffer containing 1% SDS and heated at  $95^{\circ}\text{C}$  for 5 min, followed by dilution in 4 volumes of cold lysis buffer containing 2% Triton X-100. Insoluble materials were removed by centrifugation at  $100,000 \times g$  for 10 min. Protein A-agarose (Upstate Biotechnology, Inc.)/anti-NR2A/B or anti-phosphotyrosine antibody mixtures (3  $\mu\text{g}$  each) were added to the supernatant and incubated with gentle agitation overnight at  $4^{\circ}\text{C}$ . Immunoreactive complexes were recovered by brief centrifugation; the pellets were washed first with lysis buffer (containing 2% Triton X-100) and then with phosphate-buffered saline. Proteins were finally eluted in  $2 \times$  sample buffer (1  $\times$  sample buffer consists of 2% SDS, 50 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue) and heated at  $95^{\circ}\text{C}$  for 5 min. Immunoprecipitated proteins were subjected to SDS-PAGE using 8% polyacrylamide gels (33).

Proteins were transferred onto nitrocellulose membranes as described by Towbin *et al.* (34). Western blots were performed as described previously with the following antibodies: anti-NR2A/B antibodies that recognize the C-terminal domain of NR2A and NR2B subunits of the NMDA receptors (1:200; Chemicon,); anti-phosphotyrosine antibodies (0.5–2  $\mu\text{g}/\text{ml}$ ; Upstate Biotechnology, Inc.). Subunit-specific antibodies for the immunoprecipitation and immunoblotting of NR2A and NR2B were purchased from Chemicon. They were raised against fusion proteins containing amino acid sequences from the C-terminal domains of NR2A (amino acids 1253–1391) or NR2B (amino acids 984–1104). Immunoblots were scanned, and the digitized images were quantitatively analyzed by densitometry with the ImageQuant program providing volume and apparent molecular weights. Amounts of NR2 subunits were quantified by estimating the volume of the bands labeled with the various antibodies used in the study.

**Determination of Calpain Cutting Sites in NR2A C-terminal Domain**—The 920-base pair NR2A fragment (C-terminal domain from residues 1159 to 1464) was generated by reverse transcriptase-polymerase chain reaction from rat brain RNA as described in Ferhat *et al.* (35) with the following primers: upper primer, ATATATGAATTCCGCAAGGGGACTCCACTG; lower primer, CTATTAGTCGACTATAAACATCAGATTGACTAGG; nucleotide positions 3697–3717 and 4594–4617, respectively, are based on sequence M91561. Forward and reverse primers contained an *EcoRI* and a *SalI* site, respectively, in 5' (italics in the primer sequences). Polymerase chain reactions were performed using in-house  $10 \times$  buffer (250 mM Tris, 500 mM KAC (pH 9.0)), 10% glycerol, 1.5 mM  $\text{MgCl}_2$ , 0.8 mM dNTPs, 200 nM primers, 1 unit of a 10:1 unit ratio of *rTaq/rPfu* DNA polymerases (Perkin-Elmer and Stratagene, respectively) per 50- $\mu\text{l}$  reaction. The polymerase chain reaction fragments were subcloned into *EcoRI* and *SalI* sites of pGEX-4T-1 (Amersham Pharmacia Biotech) predigested with the same enzymes. Following transformation in *Escherichia coli* BL21 (Stratagene), the constructs were sequenced on both strands. Expression of the GST-NR2A fusion proteins or GST alone (negative control) was induced with isopropyl-1-thio- $\beta$ -D-galactopyranoside, and bacteria were harvested and lysed in ice-cold phosphate-buffered saline by three freeze-thaw cycles followed by sonication. The lysed bacterial extract was centrifuged at  $12,000 \times g$  for 20 min. The supernatants were incubated with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) for 1 h. GST and NR2A-GST fusion proteins bound to the resin were washed and then eluted with glutathione elution buffer (20 mM glutathione, 100 mM

Tris-HCl (pH 8.0), 120 mM NaCl). NR2A-GST fusion proteins were incubated with calpain and calcium for 15 min at  $37^{\circ}\text{C}$ , and the proteins were then subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After staining with 0.1% Coomassie Blue R-250, one band (relative molecular mass of 20 kDa) was found in calpain-treated samples that was also recognized by anti-NR2 antibodies, and this band was subjected to protein sequence.

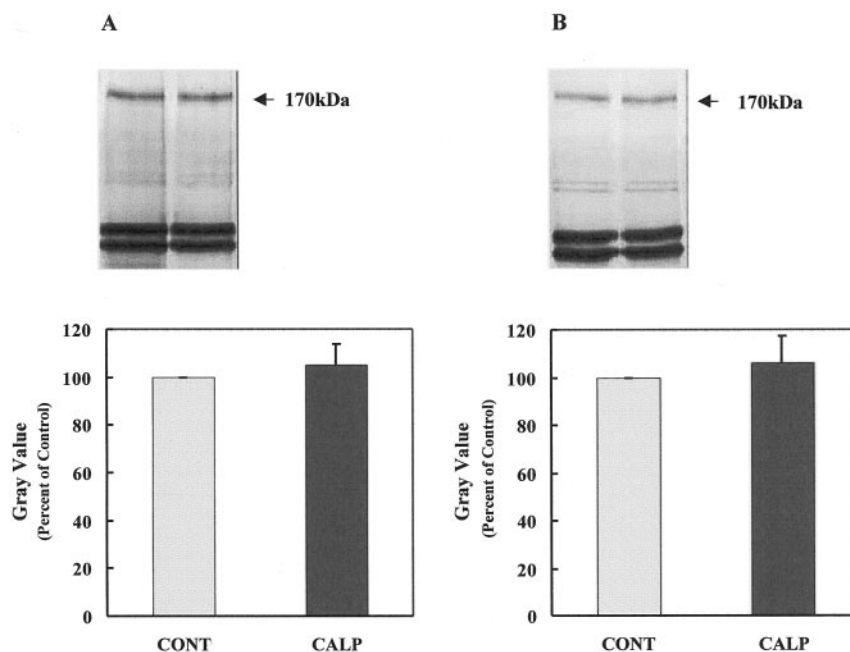
**Phosphorylation and Truncation of Recombinant NR2A Proteins**—NR2A-GST fusion protein was phosphorylated by Src (Upstate Biotechnology, Inc.) for 60 min at  $30^{\circ}\text{C}$ . Phosphorylation reaction buffer contained 100 mM Tris-HCl (pH 7.2), 5 mM  $\text{MgCl}_2$ , 2.5 mM  $\text{MnCl}_2$ , 1 mM dithiothreitol, 500  $\mu\text{M}$  ATP with or without Src (0.25 unit/ $\mu\text{l}$ ). The reaction mixture was heated at  $56^{\circ}\text{C}$  for 30 min to inactivate Src. Control and Src-treated fusion proteins were then incubated with calpain (5 ng/ $\mu\text{l}$ , 0.6 units/ml) and  $\text{Ca}^{2+}$  (2 mM) for 15 min at  $37^{\circ}\text{C}$ . After denaturing proteins in sample buffer, aliquots were subjected to SDS-PAGE (10% polyacrylamide) and transferred onto nitrocellulose membranes. Blots were incubated overnight with an anti-NR2A/B antibody (Chemicon) or an anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.). NR2A fusion protein was detected using alkaline phosphatase-conjugated secondary antibody (Bio-Rad). Blots were scanned and analyzed by using ImageQuant software.

#### RESULTS

**Effects of Calpain Treatment of Synaptic Membranes on Phosphorylated and Unphosphorylated NR2 Subunits of NMDA Receptors**—Western blots prepared from proteins immunoprecipitated by anti-phosphotyrosine antibodies exhibited a band with an apparent molecular mass of 170 kDa when stained with antibodies against either phosphotyrosine (Fig. 1A) or NR2A/B (Fig. 1B). Thus, NR2 subunits of NMDA receptors in synaptic membranes are, at least partially, phosphorylated at tyrosine residues as previously reported (35). Calpain treatment of synaptic membranes did not produce significant changes in the 170-kDa band stained with either anti-phosphotyrosine or anti-NR2A/B antibodies in immunoblots of proteins immunoprecipitated by anti-phosphotyrosine antibodies (Fig. 1 and Table I). When anti-phosphotyrosine and anti-NR2A/B antibodies were used to label immunoblots prepared from proteins precipitated with anti-NR2A/B antibodies (Fig. 2), both antibodies labeled a band with an apparent molecular mass of 170 kDa. In agreement with our previous results (26), incubation of synaptic membranes with purified calpain I produced a large decrease (62%) in the 170-kDa band stained with anti-NR2A/B antibodies following immunoprecipitation with anti-NR2A/B antibodies (Fig. 2 and Table I), indicating calpain-mediated truncation. However, only a small decrease was observed when immunoblots of the same samples were stained with anti-phosphotyrosine antibodies (Fig. 2 and Table I).

To determine whether calpain-mediated truncation of NR2A and NR2B was differentially regulated by tyrosine phosphorylation, similar experiments were performed using anti-NR2A, anti-NR2B, and anti-phosphotyrosine antibodies. Immunoblots of proteins precipitated by anti-phosphotyrosine antibodies and stained with anti-NR2A or anti-NR2B antibodies revealed bands with apparent molecular masses of 170 and 180 kDa, respectively (not shown). Calpain treatment of synaptic membranes did not produce significant changes in either the 170-kDa band stained with anti-NR2A antibodies or in the 180-kDa band stained with anti-NR2B antibodies (Table I). When anti-phosphotyrosine and anti-NR2A antibodies were used to label immunoblots of proteins precipitated with anti-NR2A antibodies, both antibodies labeled a band with an apparent molecular mass of 170 kDa (Fig. 3). Incubation of synaptic membranes with purified calpain I produced a large decrease (55%) in the 170 kDa stained with anti-NR2A antibodies following immunoprecipitation with anti-NR2A antibodies (Fig. 3), indicating calpain-mediated truncation of NR2A subunits. However, no significant decrease in the 170-kDa band was observed when immunoblots were stained with anti-phosphotyrosine antibodies

**FIG. 1. Effect of calpain treatment on tyrosine-phosphorylated NR2A/B.** Synaptic membranes were treated with calpain, and the samples were immunoprecipitated with anti-phosphotyrosine antibodies as described under "Experimental Procedures." Precipitated proteins were then processed for immunoblotting and staining with anti-phosphotyrosine (A) or anti-NR2A/B (B) antibodies. *Top panel*, representative images of Western blots, with control samples in *left lanes* and calpain-treated samples in *right lanes*. *Arrows* indicate the molecular mass of NR2 subunits. *Lower panel*, quantification of immunoblots. The amount of the 170-kDa band was quantified by integrating the volume as described under "Experimental Procedures." Results are means  $\pm$  S.E. of three determinations.



**TABLE I**  
*Effects of calpain on tyrosine-phosphorylated versus non-phosphorylated NR2 subunits of NMDA receptors*

Synaptic membranes were incubated in the absence or presence of calpain as described under "Experimental Procedures." NMDA receptors were immunoprecipitated with anti-phosphotyrosine, anti-NR2A/B, anti-NR2A, or anti-NR2B antibodies. Precipitated proteins were processed for immunoblots and the blots stained with the indicated antibodies. The amounts of NR2 subunits were determined by scanning the immunoblots, and the results were expressed as percentage of the control values. Results are means  $\pm$  S.E. of three experiments.

Staining	Immunoprecipitation			
	Anti-phosphotyrosine	Anti-NR2A/B	Anti-NR2A	Anti-NR2B
		%		
Anti-phosphotyrosine	105 $\pm$ 10	88 $\pm$ 2	92 $\pm$ 6	92 $\pm$ 5
Anti-NR2A/B	106 $\pm$ 12	38 $\pm$ 13		
Anti-NR2A	89 $\pm$ 3		45 $\pm$ 8	
Anti-NR2B	99 $\pm$ 1			70 $\pm$ 9

ies (Fig. 3 and Table I). When anti-NR2B antibodies were used to label immunoblots of proteins precipitated with anti-NR2B antibodies (Fig. 4), there was also a decrease in the 180-kDa band labeled with anti-NR2B antibodies. However, this decrease was much less than that observed for NR2A (30%). Furthermore, in this case, an additional band with an apparent molecular mass of about 150 kDa appeared following calpain treatment (Fig. 4A, *arrow*); the amount of the 150 kDa represented about 30% of the control 180-kDa band, indicating that the decrease in the 180-kDa band was due to the formation of the 150-kDa band. In addition, calpain treatment of synaptic membranes did not result in significant changes in the 180-kDa band stained with anti-phosphotyrosine (Fig. 4B and Table I).

**Identification of Calpain Cutting Sites in NR2A C-terminal Domain**—A GST-NR2A fusion protein was expressed in bacteria and used to identify possible calpain cutting sites in the C-terminal domain. Calpain treatment of the fusion protein resulted in the formation of several lower molecular weight bands. A 20-kDa band was recognized by anti-NR2 antibodies and was sequenced by using a gas-phase protein sequencer. The sequencing of the N-terminal domain of this band provided the following sequence: FQKNKLRINR. After matching with

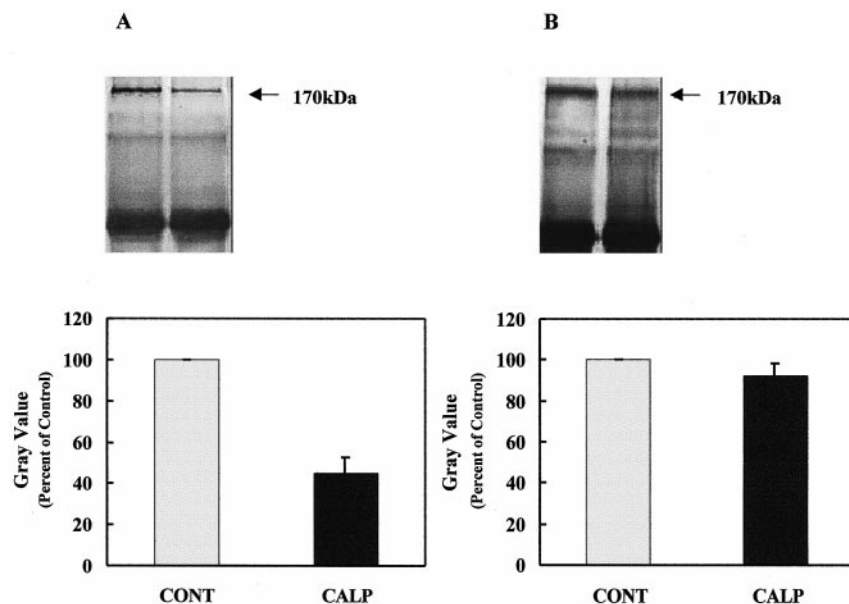
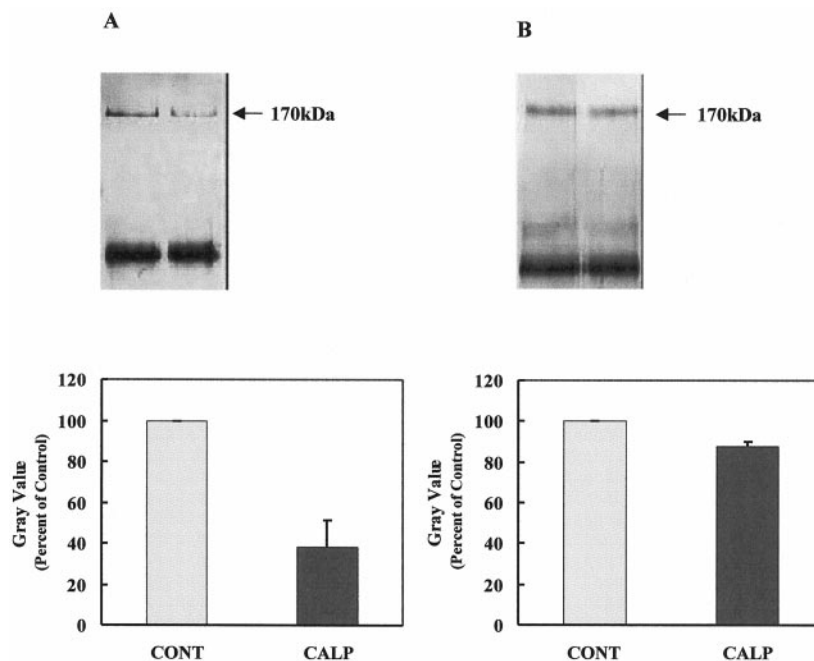
GenBank™, this sequence was found to be identical with a sequence in the C-terminal domain of rat NR2A subunit of NMDA receptors. It indicated the existence of a major cutting site in the NR2A C-terminal domain between residues 1278 and 1279 (Fig. 5). Interestingly, such a site corresponds to a preferred cutting site for calpain (25).

**Effect of Src-mediated Phosphorylation on Calpain Truncation of NR2A Fusion Protein**—To verify the effect of the state of tyrosine phosphorylation of NR2A receptor on calpain-mediated truncation, NR2A fusion proteins were first preincubated in the presence or absence of Src or ATP, before the incubation with purified calpain I. We first incubated NR2A fusion protein with increasing concentrations of calpain to select a concentration of calpain that would result in a relatively large degree of truncation (Fig. 6A). Incubation of NR2A fusion protein with 5 ng/ $\mu$ l calpain for 15 min at 37 °C resulted in a 68% decrease in the band recognized by anti-NR2A/B antibodies (Fig. 6, B and C). Preincubation of NR2A fusion protein with Src and ATP significantly reduced calpain-mediated truncation. Staining of the same blots with anti-phosphotyrosine antibodies confirmed that, under these conditions, Src incubation produced a large enhancement of tyrosine phosphorylation of NR2A fusion protein (Fig. 6B). Src itself did not interfere with NR2A truncation by calpain (Fig. 6, B and C). Omitting ATP from the incubation mixture also abolished the effects of Src, indicating that Src-mediated phosphorylation of NR2A fusion protein is required for the protective effect.

## DISCUSSION

Our results clearly indicate that tyrosine phosphorylation almost completely protects NR2A and NR2B subunits of NMDA receptors from calpain-mediated truncation of their C-terminal domains. Thus, immunoprecipitation with anti-phosphotyrosine did precipitate NR2 subunits of NMDA receptors as previously reported by many laboratories (36, 37). No evidence for calpain-mediated truncation of tyrosine-phosphorylated NMDA receptor subunits was obtained. Under the same conditions, immunoprecipitation with anti-NR2A/B, anti-NR2A, and anti-NR2B indicated that calpain treatment produced a 62% decrease in the amount of NR2A/B, a 55% decrease in NR2A, and a 30% decrease in NR2B. The reason for the difference in percent decrease between anti-NR2A/B and the average of the values for anti-NR2A and anti-NR2B is not

**FIG. 2. Effects of calpain treatment on total and phosphorylated NR2A/B.** Synaptic membranes were treated with calpain, and the samples were immunoprecipitated with anti-NR2A/B antibodies as described under "Experimental Procedures." Precipitated proteins were then processed for immunoblotting and staining with anti-NR2A/B (A) or anti-phosphotyrosine (B) antibodies. *Top panel*, representative images of Western blots, with control samples in *left lanes* and calpain-treated samples in *right lanes*. *Arrows* indicate the molecular mass of NR2 subunits. *Lower panel*, quantification of immunoblots. The amount of the 170-kDa band was quantified by integrating the volume as described under "Experimental Procedures." Results are means  $\pm$  S.E. of three determinations.



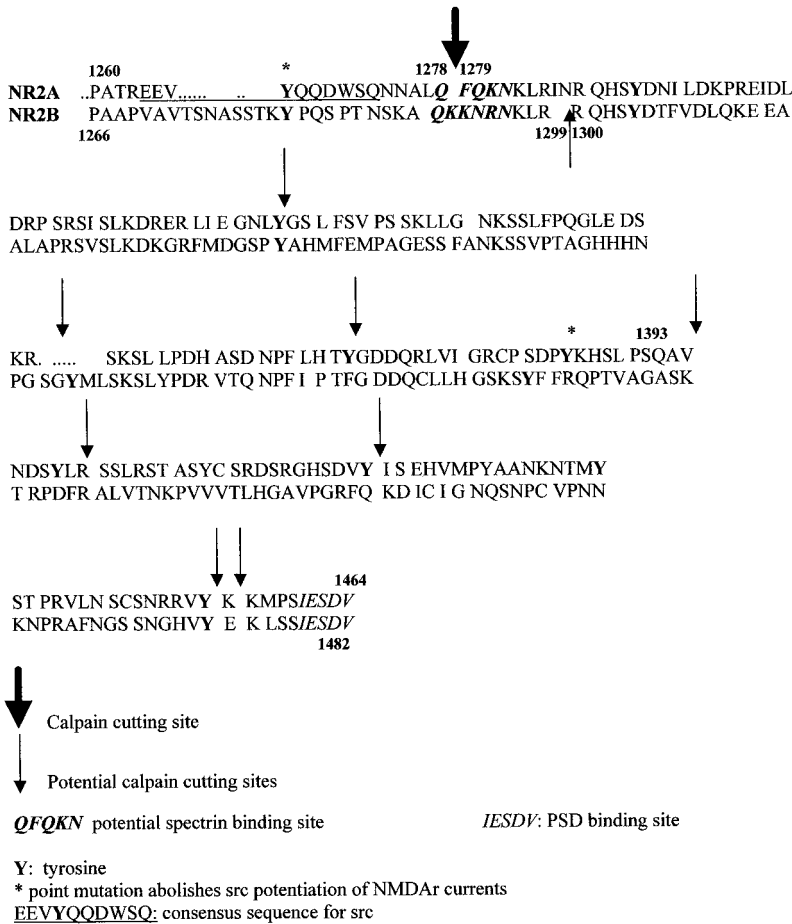
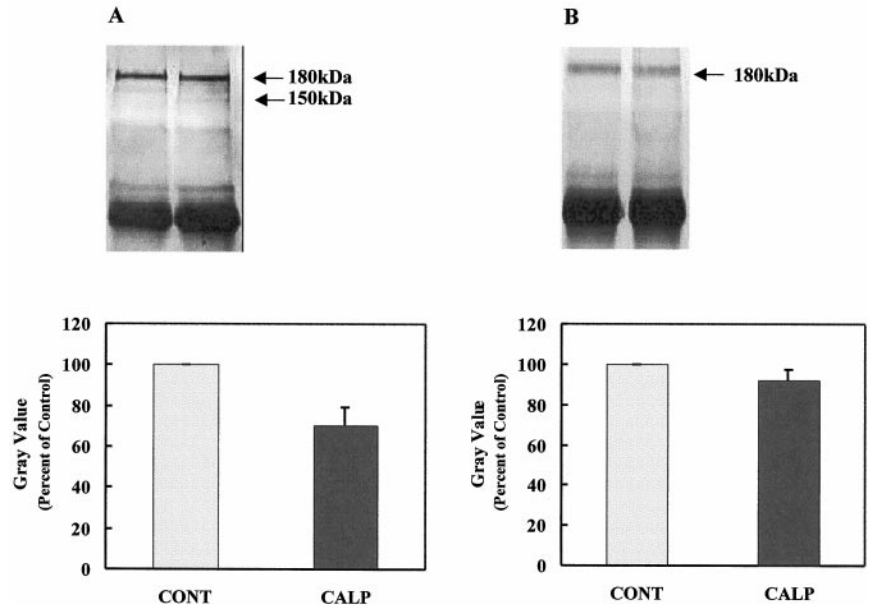
**FIG. 3. Effects of calpain treatment on total and phosphorylated NR2A.** Synaptic membranes were treated with calpain, and the samples were immunoprecipitated with anti-NR2A antibodies as described under "Experimental Procedures." Precipitated proteins were then processed for immunoblotting and staining with anti-NR2A (A) or anti-phosphotyrosine (B) antibodies. *Top panel*, representative images of Western blots, with control samples in *left lanes* and calpain-treated samples in *right lanes*. *Arrows* indicate the molecular mass of NR2 subunits. *Lower panel*, quantification of immunoblots. The amount of the 170-kDa band was quantified by integrating the volume as described under "Experimental Procedures." Results are means  $\pm$  S.E. of three determinations.

clear and could be due to differences in the ability of the antibodies to precipitate the subunits. The same samples exhibited only small or no decreases in the 170/180-kDa species labeled with anti-phosphotyrosine antibodies, providing further evidence that the tyrosine-phosphorylated forms of the 170–180-kDa species of NR2A and NR2B were not degraded by calpain. The exact fraction of tyrosine-phosphorylated NR2A or NR2B is not clearly known. It was estimated that only a small percentage of subunits was phosphorylated under control conditions and that this fraction could be markedly increased by different manipulations (36). It is thus not likely that the calpain-resistant fraction of NR2 subunits (45% for NR2A and 70% for NR2B) represents the fraction of tyrosine-phosphorylated subunits. Other factors must contribute to resistance to calpain-mediated truncation of the subunits including differences in sequences, other phosphorylation sites, or other post-translational modifications.

Using a GST-NR2A fusion protein provided the identification of one calpain cutting site in the NR2A C-terminal domain

between residues 1278 and 1279 (Fig. 5). Other potential cutting sites exist for both NR2A and NR2B and have been indicated in the figure. These sites have been identified by comparison with known cutting sites for several calpain substrates as well as with a variety of peptides shown to be degraded by calpain (25). First, it is clear that there are many more potential calpain cutting sites in the C-terminal domain of NR2A than of NR2B. This might account for the larger decrease in NR2A than NR2B following calpain treatment of membranes. Second, the antibodies used to recognize NR2A/B are directed at the last 10 amino acids of the C-terminal domain, and it is thus likely that some fragments generated by calpain-mediated truncation of the subunits would not easily be observed in immunoblots. Interestingly, the anti-NR2B antibodies are directed at amino acids 984–1104 and would therefore still recognize receptor subunits lacking the C-terminal domain if the truncation site(s) is located after amino acid 1104. One potential cutting site at amino acids 1299–1300 is present in the C-terminal domain of NR2B. This site could generate the lower

**FIG. 4. Effects of calpain treatment on total and phosphorylated NR2B.** Synaptic membranes were treated with calpain, and the samples were immunoprecipitated with anti-NR2B antibodies as described under "Experimental Procedures." Precipitated proteins were then processed for immunoblotting and staining with anti-NR2B (A) or anti-phosphotyrosine (B) antibodies. *Top panel*, representative images of Western blots, with control samples in *left lanes* and calpain-treated samples in *right lanes*. Arrows indicate the molecular mass of NR2 subunits. *Lower panel*, quantification of immunoblots. The amount of the 180-kDa band was quantified by integrating the volume as described under "Experimental Procedures." Results are means  $\pm$  S.E. of three determinations. Note the appearance of a new band with an apparent molecular mass of 150 kDa following calpain treatment (A).

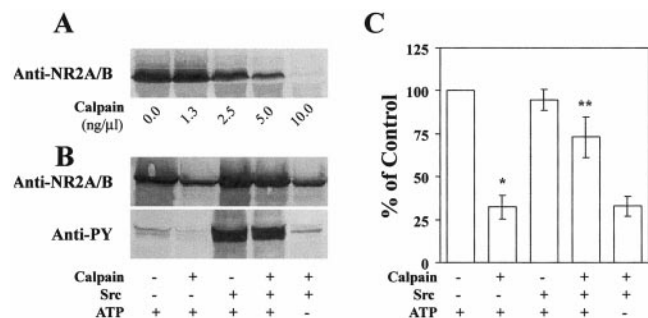


**FIG. 5. Amino acid sequences of the C-terminal domains of NR2A and NR2B.** The wide arrow indicates the demonstrated calpain cutting sites, and the narrow arrows indicate potential calpain cutting sites (25). Bold Ys indicate tyrosine sites, and \* indicates tyrosine sites where point mutations abolish Src potentiation of NMDA receptor currents (18). The underlined sequence corresponds to a consensus sequence for Src phosphorylation. Also indicated in the sequence is the potential spectrin-binding site in NR2B (37).

molecular weight species of NR2B observed in the immunoblot shown in Fig. 4. In particular, a species with an apparent molecular mass of about 150 kDa increased significantly following calpain treatment of membranes. The antibodies against NR2A are directed at amino acids 1253–1391 and would therefore still recognize receptors lacking only the last 75 amino acids of the subunit or subunits truncated before amino acid 1253. No small or large fragments were detected in immunoblots stained with anti-NR2A antibodies. This would

be consistent with cutting sites located between amino acids 1253 and 1391.

There are many tyrosine residues in the C-terminal domains of both NR2A and NR2B (indicated by bold letters in Fig. 5). Although the exact location of the tyrosine(s) phosphorylated in either NR2A or NR2B are not yet known, 2 tyrosine residues (amino acid 1267 and amino acid 1387) have been identified in the C-terminal domain of NR2A, the mutations of which prevent Src-mediated increase in NMDA receptor function (18).



**FIG. 6. Effect of Src phosphorylation of NR2A fusion protein on calpain-mediated truncation.** *A*, NR2A fusion protein was incubated with increasing concentrations of calpain for 15 min at 37 °C. Aliquots were processed for SDS-PAGE and Western blots using anti-NR2A/B antibodies. *B*, NR2A fusion protein was preincubated in the absence or presence of Src and ATP for 60 min at 30 °C followed by heating at 56 °C for 30 min to inactivate Src. The reaction mixtures were then incubated for an additional 15 min at 37 °C with or without calpain and calcium. Aliquots were processed for Western blots using anti-phosphotyrosine or anti-NR2A/B antibodies. *C*, quantification of Western blots similar to those shown in *B*. The amount of the NR2A fusion protein in immunoblots stained with anti-NR2A/B antibodies was quantified. Data were expressed as percent of control and represent the means  $\pm$  S.D. of three experiments. \*, statistically significant difference between control and calpain; \*\*, statistically significant difference between calpain and calpain and Src (Student's *t* test).

The former site is interesting, as it is closely located from the calpain-cutting site at amino acids 1278–1279. Although our data did not provide for the identification of which tyrosine residue(s) is involved in the protection from calpain-mediated truncation, they did confirm that Src-mediated phosphorylation of the C-terminal domain of NR2A inhibits calpain-mediated truncation. More information is needed to understand better the mechanisms responsible for protection against calpain-mediated truncation of NMDA receptors by tyrosine phosphorylation. However, it is worth noting that this feature, which is phosphorylation-mediated protection, is a common feature of calpain substrates (30).

Another interesting feature of the potential calpain cutting sites concerns their relationships to a spectrin-binding site recently reported to be present in the C-terminal domains of NR2A and NR2B. For NR2B, the site has been proposed to be at the sequence QKKNRN (amino acids 1291–1296) (37), which is at a site located very close to a potential calpain cutting site (amino acids 1299–1300). Although no information is available for the exact location of the spectrin-binding sites of NR2A, if we assume that it is similar to that in NR2B and corresponds to the sequence QFQKN (amino acids 1278–1282), this would overlap with the calpain cutting site (amino acids 1278–1279). It is worth noting that spectrin itself is a preferred calpain substrate, and therefore both NR2 subunits and spectrin could be truncated as a result of calpain activation. Besides spectrin, the C-terminal regions of the NR2 subunits of the NMDA receptors interact with the PDZ domains of a family of synaptic cytoskeleton-associated proteins that may be involved in the formation of macromolecular signaling complexes (reviewed in Refs. 38 and 39). Mice expressing C-terminal truncated NR2 subunits phenotypically resemble NR2 knock-out mice. However, C-terminal truncation of the NR2 subunits does not interfere with the formation of NMDA receptors that can be synaptically activated, leading to the hypothesis that it is rather the intracellular signaling that is deficient in cells expressing truncated receptor subunits (40). It is therefore tempting to propose that one possible function of calpain-mediated truncation of NMDA receptor subunits is to produce the disassociation of NMDA receptors from the spectrin meshwork or from the macromolecular signaling complexes. This could lead

to the relocation of the receptors in synaptic membranes to altered signal transduction pathways or be a signal for internalization and further degradation of the receptors. Experiments are underway to test this hypothesis.

Finally, it is worth considering the potential functional consequences of the interactions between calpain and tyrosine phosphorylation at the level of the C-terminal domains of NR2 subunits of the NMDA receptors. In particular, it has been shown that LTP is accompanied by increased phosphorylation of NR2 subunits of NMDA receptors (21, 22). As LTP has also been shown to be accompanied by calpain activation (41, 42), it is conceivable that one of the functions of tyrosine phosphorylation of NMDA receptors is to prevent calpain-mediated truncation of the C-terminal domains of NR2 subunits and possible down-regulation of NMDA receptors. In the absence of such a mechanism, each event leading to LTP could result in modification of the synaptic plasticity machinery itself. Similarly, ischemia results in a large increase in tyrosine phosphorylation of NR2A (at least) subunits (43). The same insult also results in calpain activation as evidenced by increased formation of calpain-mediated breakdown of the cytoskeletal protein spectrin (44, 45). Our results then predict that tyrosine phosphorylation of NR2 subunits would protect the subunits from calpain-mediated truncation of the C-terminal domains, and possibly then, from their dissociation with cytoskeletal proteins. As a result, this mechanism could maintain a higher degree of activity of NMDA receptors, despite the activation of calpain. It would be of interest to determine the effects of tyrosine kinase inhibitors on NMDA receptor locations and functions under these conditions.

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