Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin

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The *N*-methyl-D-aspartate receptor (NMDA-R) and brain spectrin, a protein that links membrane proteins to the actin cytoskeleton, are major components of post-synaptic densities (PSDs). Since the activity of the NMDA-R channel is dependent on the integrity of actin and leads to calpain-mediated spectrin breakdown, we have investigated whether the actin-binding spectrin may interact directly with NMDA-Rs. Spectrin is reported here to interact selectively *in vitro* with the C-terminal cytoplasmic domains of the NR1a, NR2A and NR2B subunits of the NMDA-R but not with that of the AMPA receptor GluR1. Spectrin binds at NR2B sites distinct from those of α -actinin-2 and members of the PSD95/SAP90 family. The spectrin–NR2B interactions are antagonized by Ca^{2+} and fyn-mediated NR2B phosphorylation, but not by Ca^{2+} /calmodulin (CaM) or by Ca^{2+} /CaM-dependent protein kinase II-mediated NR2B phosphorylation. The spectrin–NR1 interactions are unaffected by Ca^{2+} but inhibited by CaM and by protein kinase A- and C-mediated phosphorylations of NR1. Finally, in rat synaptosomes, both spectrin and NR2B are loosened from membranes upon addition of physiological concentrations of calcium ions. The highly regulated linkage of the NMDA-R to spectrin may underlie the morphological changes that occur in neuronal dendrites concurrently with synaptic activity and plasticity.

Keywords: calcium/cytoskeleton/NMDA receptors/phosphorylation/spectrin

Introduction

Long-term potentiation (LTP) is currently the most widely accepted model of activity-dependent synaptic plasticity, and growing evidence suggests that it may represent the biochemical and cellular basis for some types of memory and learning (Maren and Baudry, 1995; Cain, 1997). Concurrently with the progression of LTP, it has been found that morphometric changes take place in dendritic spines. It was proposed that these changes may form the long lasting traces of the specific patterns of neuronal activity (Fifková and Morales, 1992; Papa *et al.*, 1995; Papa and Segal, 1996). At the molecular level, the actin-based cytoskeleton and its associated proteins are likely candidates to contribute to these structural changes since they form dynamic interactions with the plasma membrane (Fifková and Morales, 1992; Hitt and Luna, 1994). The

versatility of these interactions is achieved by the sensitivity of the actin-based matrix to changes in calcium and phosphorylation states of its components. These properties allow the actin network to respond to signals transmitted by plasma membrane constituents. Changes in the actin cytoskeleton, in turn, may trigger a chain of biochemical events which outlasts the initial signal and leads to long-term modifications (Fifková and Morales, 1992).

In the processes of LTP and synaptic plasticity, the glutamate receptors of the *N*-methyl-D-aspartate (NMDA) subtype play a central role (Collingridge and Bliss, 1995). It has been suggested that part of the molecular machinery of synaptic plasticity is based on a link between the NMDA receptor (NMDA-R) and the neuronal cytoskeleton. Several experimental findings support this suggestion: (i) among the ionotropic glutamate receptors, the NMDA-R is the only one found to be mechanosensitive (Paoletti and Ascher, 1994); (ii) most of the NMDA-Rs expressed on the soma and dendrites of living visual cortex neurons are immobile, suggesting anchorage by cytoskeletal elements (Benke *et al.*, 1993); (iii) the repetitive activation of the NMDA-R channel induces a decrease in macroscopic conductance also called 'run-down'. This phenomenon is dependent on Ca^{2+} ions, can be mimicked by cytochalasin which promotes actin depolymerization, and can be prevented by phalloidin, an inhibitor of actin depolymerization. The 'run-down' of the NMDA-R channels may thus reflect a detachment of actin-binding proteins that presumably link these receptors to the actin cytoskeleton. Such proteins may be affected directly by calcium ions or modulated by calcium-dependent processes (Rosenmund and Westbrook, 1993a,b).

Although the existence of such linker proteins is supported by the available data, their identity is still obscure. Brain spectrin could be one of these linker proteins. Notably, spectrin connects integral membrane proteins to the actin filaments (Morrow, 1989; Bennett and Gilligan, 1993; Davis and Bennett, 1994; Lombardo *et al.*, 1994) and its interactions with the plasma membrane are known to be regulated by calcium, either directly or through calmodulin (CaM) (Steiner *et al.*, 1989; Backman, 1996). In addition, calcium influx through activated NMDA-Rs induces spectrin breakdown via calpain (Seubert *et al.*, 1988; Di Stasi *et al.*, 1991; Faddis *et al.*, 1997), a protease whose activity is linked to the progression of LTP (Lynch and Baudry, 1987; Vanderklish *et al.*, 1995). Since, in addition to the above, biochemical and electron microscopy data show that both NMDA-Rs and spectrin localize in post-synaptic densities (PSDs) (Zagon *et al.*, 1986; Fifková and Morales, 1992; Malchiodi-Albedi *et al.*, 1993; Petralia *et al.*, 1994a,b; Kennedy, 1997), we investigated here whether spectrin and NMDA-R directly interact. The results presented below establish that spectrin–NMDA-R interactions not only occur, but are extensively regulated

Fig. 1. Binding of brain spectrin to NMDA-R subunits. Purified MBP fusion proteins consisting of the cytoplasmic C-terminal domain of various glutamate receptor subunits were subjected to SDS-PAGE followed by Western blotting. (A) Proteins were detected by Ponceau-red staining and the Western blot was then incubated in overlay buffer followed by incubation with [125 I]spectrin (1 μ g/ml) in overlay buffer. (B) Following washing, [125 I]spectrin-binding proteins were detected using the 445 SI PhosphorImager (Molecular Dynamics). Lane 1, MBP-NR1 [amino acids 834–938]; lane 2, MBP-NR2A [838–1464]; lane 3, MBP-NR2B [1086–1481]; lane 4, MBP-GluR1 [827–908]; lane 5, MBP- β -gal α -fragment.

by plasticity-related factors. Thus, spectrin binding to the NMDA-R may possibly form the basis for the actin-mediated regulation of the NMDA-R channel activity and represent one of the events leading to the plasticity-induced changes in spine morphology.

Results

Direct binding of spectrin to NMDA-R subunits

In order to demonstrate a direct association between brain spectrin and the NMDA-R subunits, fusion proteins between the maltose-binding protein (MBP) and the cytoplasmic C-terminal domains of the NR1, NR2A and NR2B subunits were purified by affinity chromatography, fractionated by SDS-PAGE, blotted to nitrocellulose (NC) filters and subjected to filter overlay assay using [125 I]spectrin. The Ponceau-red staining of the NC filter is shown in Figure 1A. It indicates that approximately equal amounts of protein are present from each fusion protein. The right panel of Figure 1B shows [125 I]spectrin binding to the cytoplasmic C-terminal domains of NR1, NR2A and NR2B (lanes 1, 2 and 3, respectively) but not to the cytoplasmic domain of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR1 or the MBP- β -gal α -fragment (lanes 4 and 5, respectively).

'Pull-out' of spectrin from brain cytosol by NR1 and NR2B cytoplasmic domains

To confirm further the specificity of the spectrin-NMDA-R interaction, fusion proteins between GST and the cytoplasmic C-terminal domains of NR1 and NR2B subunits or GST alone were immobilized separately on a glutathione affinity matrix. Brain cytosolic fractions in a native form or denatured by boiling were applied batchwise to the affinity matrices. The bound material was eluted from the matrices, fractionated by SDS-PAGE, immunoblotted and analysed with anti-spectrin antibodies. Figure 2 shows that brain spectrin is associated with both the NR1 and NR2B cytoplasmic C-terminal domains. The binding is specific since no spectrin is bound to the control GST. Similarly, no binding is detected when heat-denatured

Fig. 2. 'Pull-out' of spectrin from crude brain cytosol extracts by GST fusion proteins with NR1 (amino acids 834–938) and NR2B (1086–1481) C-terminal domains. GST fusion proteins were purified from bacterial extracts on glutathione-Sepharose beads and incubated overnight at 4°C with either native (n) or boiled (b) brain cytosol. Following washes, the bound proteins were eluted from the beads with SDS sample buffer (Laemmli, 1970), separated by SDS-PAGE and analysed on Western blot with anti-spectrin antibodies. The total input of the crude cytosolic extract (200 μ g) is also shown (extract).

extract is offered to the fusion protein-bound resin. The above results (Figures 1 and 2) indicate that brain spectrin interacts directly and selectively with the NR1, NR2A and NR2B C-terminal domains of the NMDA-R. This set of interactions is wider than that formed by α -actinin-2, which binds to NR1 and NR2B but not to NR2A (Wyszynski *et al.*, 1997).

Partial localization of the spectrin-binding site in NR2B

In order to localize the spectrin-binding domain of the NR2B subunit, fragments of the NR2B C-terminal domain were prepared as MBP fusion proteins (Figure 3A). When designing the fragments, overlaps of 15–20 amino acids were introduced to minimize loss of binding due to splitting of possible binding sites. The MBP fusion proteins were subjected to [125 I]spectrin overlay as described earlier. The Ponceau-red staining shows the amounts of protein for each fusion protein (Figure 3B, top). The [125 I]spectrin binding is shown at the bottom of Figure 3B. The ratios of spectrin binding per amount of protein were calculated for each fragment by densitometry, with the value for the K1086–V1481 fragment normalized to 100% (Figure 3C). Brain spectrin bound to K1086–V1481 (Figure 3, lane 1), and deletion of 155 amino acids from the C-terminal side did not prevent its binding to K1086–D1326 (Figure 3, lane 2). Spectrin did not bind to the V1308–V1481 region (Figure 3, lane 3). Since both the α -actinin-2- and PSD-95/SAP102-binding sites reside in this region (Lau *et al.*, 1996; Müller *et al.*, 1996; Wyszynski *et al.*, 1997), this finding suggests that the latter three proteins and brain spectrin bind to distinct regions in NR2B. Inspection of the amino acid sequence of the K1086–D1326 fragment reveals a putative spectrin-binding motif (Herrera *et al.*, 1993; Iida *et al.*, 1994), KKNXN (K1292–N1296), and a putative SH3 minimal binding domain, PXXP (P1114–1117), that may bind the spectrin SH3 domain (Noble *et al.*, 1993; Viguera *et al.*, 1996). In order to define further the spectrin-binding site of NR2B, the K1086–D1326 fragment was dissected into two smaller fragments, K1086–S1226 and V1204–D1326. The K1086–S1226 fragment, which contains the PXXP motif, exhibited weak spectrin binding (Figure 3, lane 4), while the V1204–D1326 fragment, which contains the KKNXN motif, retained significant spectrin binding. Nevertheless, the sum of spectrin binding to the two smaller fragments was 20% smaller than its binding to the parent fragment (K1086–D1326) (Figure 3, lane 5). These results suggest

Fig. 3. Localization of the spectrin-binding domain of NR2B. (A) Fusion proteins of MBP with various fragments of the NR2B C-terminal domain were prepared. Overlaps were introduced in order to cover the possible loss of spectrin-binding sites at the junctions. The fusion proteins were subjected to SDS-PAGE followed by Western blotting. (B) Proteins were detected by Ponceau-red staining (top) and the Western blot was then incubated in overlay buffer followed by incubation with [125 I]spectrin (1 μ g/ml) in overlay buffer. Following washing, [125 I]spectrin-binding proteins were detected using the PhosphorImager (bottom). The Ponceau-red-stained bands as well as the 125 I-labelled bands were analysed by densitometry using the NIH image 1.55TL program. Their ratio is expressed as percentage spectrin-binding and shown in (C). The extent of binding of [125 I]spectrin to fragment 1 was set as 100%.

that spectrin may bind to multiple sites in NR2B and that a particular folding of the NR2B cytoplasmic domain creates a specific spectrin-binding site. The NR2B fragments K1086–S1226 and V1204–D1326 harbour 23 and 36% sequence identity to the parallel sequences in NR2A, respectively. Thus, there is no obvious sequence conservation between these two subunits.

Phosphorylation of NR1 and NR2B inhibits spectrin binding

The NMDA-R is a major substrate for various protein kinases (Tingley *et al.*, 1993, 1997; Lieberman and Mody, 1994; Moon *et al.*, 1994; Suzuki and Okumura-Noji, 1995; Omkumar *et al.*, 1996) which greatly affect its channel activity and the key role it plays in various synaptic events including LTP (Chen and Huang, 1992; Grant *et al.*, 1992; Köhr and Seeburg, 1996; Raman *et al.*, 1996; Yu *et al.*, 1997). Accordingly, it was of interest to determine whether the phosphorylation of the NMDA-R affects its interactions with spectrin. To test the direct effects of phosphorylation on the interaction, fusion proteins containing the C-terminal domains of either NR1 or NR2B were phosphorylated *in vitro* by purified kinases in the presence of [γ - 32 P]ATP (except in the case of fyn-mediated phosphorylation which was analysed using anti-phosphotyrosine antibodies). As a control, MBP- β -gal α -fragment fusion protein was incubated with each kinase but failed to be phosphorylated (data not shown). Parallel phosphorylation reactions with unlabelled ATP were performed, and the fusion proteins were subjected to SDS-PAGE and then assayed by filter overlay for [125 I]spectrin binding. Tyrosine phosphorylation of NR2B C-terminal domain by fyn markedly inhibited spectrin binding (Figure 4A, left), whereas phosphorylation by Ca^{2+} /CaM-dependent protein kinase II (CaMKII) had no effect (Figure 4A, right). In contrast, phosphorylation of NR1 C-terminal domain by both protein kinase C (PKC) and protein kinase A (PKA) (Figure 4B) markedly inhibited spectrin binding. This finding is in accord with the previous observations that the targets of phosphorylation by PKC and PKA on NR1 are two neighbouring serine residues (Tingley *et al.*, 1997).

Fig. 4. *In vitro* phosphorylation of the NR1 and NR2B C-terminal domains affect spectrin binding. MBP fusion proteins with NR2B and NR1 C-termini (5 μ g) were phosphorylated *in vitro* by purified kinases. The phosphorylated and non-phosphorylated fusion proteins were subjected to SDS-PAGE followed by Western blotting. The blots were then incubated in overlay buffer followed by incubation with [125 I]spectrin (1 μ g/ml) in overlay buffer. Following washing, [125 I]spectrin-binding proteins were detected using the PhosphorImager. The extent of phosphorylation was verified in each case by parallel reactions using either anti-phosphotyrosine antibodies or [γ - 32 P]ATP. (A) Phosphorylation of the NR2B C-terminus by purified fyn and CaMKII. (B) Phosphorylation of the NR1 C-terminus by the purified catalytic subunits of PKC and PKA.

Effect of calcium ions on spectrin–NMDA receptor interaction

The NMDA-R activity is also regulated by calcium ions. Influx of calcium ions through NMDA-R channels leads to a fast inactivation followed by a long-lasting ‘run-down’ of the NMDA response amplitude which has been attributed to the detachment of a hypothetical actin-binding protein (Rosenmund and Westbrook, 1993a,b). We therefore tested whether calcium ions could affect the spectrin–NMDA-R interaction. [125 I]Spectrin binding to blotted NR1 and NR2B C-terminal domains was carried out in the presence of increasing concentrations of calcium

Fig. 5. (A) Calcium ions differentially affect the binding of brain spectrin to NR2B and NR1. Purified MBP fusion proteins with NR1 and NR2B C-termini were subjected to SDS-PAGE and Western blotting. The blots were then overlaid with 1 $\mu\text{g}/\text{ml}$ [^{125}I]spectrin as described above in the presence of either 1 mM EGTA (zero point) or increasing concentrations of CaCl_2 . Following washes in PBS, the bound [^{125}I]spectrin was detected by PhosphorImager and quantified by densitometry. The intensity value in each case was plotted against the respective calcium ion concentration. Each concentration was tested in triplicate. NR1 ($\square$), NR2B ($\triangle$). The insets display a typical spectrin-binding profile. (B) $\text{Ca}^{2+}/\text{CaM}$ differentially affects the binding of brain spectrin to NR1 and NR2B. [^{125}I]Spectrin overlay experiments were carried out as in (A) except that the Ca^{2+} concentration at all points was fixed at 100 μM and CaM was present at increasing concentrations. The intensity value in each case was plotted against the respective CaM concentration. Each concentration was tested in triplicate. NR1 ($\square$), NR2B ($\triangle$). The insets display a typical spectrin-binding profile.

ions. Figure 5A shows that calcium ions inhibit in a dose-dependent manner the binding of [^{125}I]spectrin to the NR2B C-terminal domain (triangles) with 50% inhibition at $\sim 100 \mu\text{M}$ Ca^{2+} , a concentration that leads to the 'run-down' of the NMDA-R channel activity. In contrast, the binding of spectrin to the NR1 C-terminal domain (squares) was calcium insensitive even at 1 mM concentration.

Effect of CaM on spectrin-NMDA-R interaction

It has been shown that CaM can inhibit the binding of brain spectrin to brain integral membrane proteins (Steiner *et al.*, 1989). To test whether the binding of spectrin to the NMDA-R can be affected by CaM , [^{125}I]spectrin binding to blotted NR1 and NR2B C-terminal domains was carried out in the presence of increasing concentrations of purified brain CaM at constant calcium ions concentration (100 μM). Figure 5B shows the dose-response inhibition by CaM of spectrin binding to NR1 C-terminal domain. Inclusion of 1 μM CaM led to 50% inhibition of spectrin binding to NR1 C-terminal domain (squares) while 10 μM CaM completely abolished it, but did not prevent spectrin from binding to NR2B C-terminal domain (triangles). The $K_i = 1 \mu\text{M}$ displayed here by CaM is identical to the value obtained for CaM inhibition of spectrin binding to synaptosomal membranes (Steiner *et al.*, 1989) and to α -actinin-2 (Wyszynski *et al.*, 1997).

Co-distribution of the NMDA-R NR2 subunits and spectrin in detergent-solubilized synaptosomal fractions

NMDA-R and spectrin are major PSD components as established by both electron microscopy and biochemical studies (Zagon *et al.*, 1986; Fifková and Morales, 1992; Malchiodi-Albedi *et al.*, 1993; Petralia *et al.*, 1994a,b; Kennedy, 1997). To determine whether spectrin co-fractionates with NMDA-Rs and can be co-solubilized in detergents, synaptosomes from rat forebrain were prepared (see Materials and methods) and probed for the presence

Fig. 6. Co-distribution of NMDA-R NR2 subunits and spectrin in detergent-solubilized synaptosomal fractions. Rat synaptosomes were solubilized by various detergents. Following centrifugation at 12 000 g for 20 min, the detergent-soluble (Sups) and detergent-insoluble (Pellets) fractions were subjected to SDS-PAGE and immunoblotting with either anti-NR2A/B or anti-spectrin antibodies. Lane 1, total synaptosomal fraction; lanes 2 and 5, 1% Triton X-100, 4°C, 60 min; lanes 3 and 6, 1% sodium deoxycholate, pH 9.0, 37°C; lanes 4 and 7, 1% SDS, 95°C, 10 min.

of spectrin and NR2 subunits. Both proteins were found as expected in the synaptosomal fraction (Figure 6, lane 1). This fraction was then exposed to either one of the three detergents, Triton X-100, sodium deoxycholate or SDS, under the conditions described in the legend of Figure 6 and centrifuged in order to separate detergent-soluble proteins from pelleted detergent-insoluble proteins. The NR2 subunits were insoluble in 1% Triton X-100 (Figure 6, lane 5) but soluble in 1% deoxycholate pH 9.0 or in 1% boiling SDS (Figure 6, lanes 3 and 4, respectively), a pattern also observed for spectrin. The NR2 subunits and spectrin, however, failed to co-immunoprecipitate from detergent-treated rat synaptosomes, either because of their insolubility under non-denaturing conditions or because of the loss of interactions in the presence of detergents. In an attempt to circumvent the latter problem, membrane cross-linking experiments using disuccinimidylsuberate (DSS) or dithiobissuccinimidylpropionate (DSP) were performed but led to the formation of detergent-insoluble high molecular aggregates which did not migrate on SDS-PAGE (data not shown).

Fig. 7. Calcium-induced detergent extractability of spectrin and NR2 subunits from synaptosomal membranes. Rat synaptosomes were pre-incubated for 60 min at 4°C in the presence of calcium ions at the indicated concentrations prior to solubilization with 1% Triton X-100. Following centrifugation at 12 000 *g* for 20 min, the Triton X-100-soluble fractions were subjected to SDS-PAGE and immunoblotting with either anti-NR1, anti-NR2A/B or anti-spectrin antibodies.

Calcium-induced detergent extractability of spectrin and NR2 subunits from synaptosomal membranes

To characterize further the association of spectrin with the NMDA-R *in situ*, rat synaptosomes were pre-incubated with calcium ions prior to solubilization by 1% Triton X-100 and the detergent-soluble fractions were subsequently probed for the presence of spectrin and NMDA-R NR1 and NR2 subunits (Figure 7). While the presence of 10 μ M calcium ions resulted in a significant detachment of spectrin from the detergent-insoluble fraction, the presence of 50 μ M calcium caused both spectrin and the NR2 subunits to be solubilized by 1% Triton X-100. This result is not only consistent with the inhibitory effect of calcium on the NR2B–spectrin interaction seen *in vitro* (Figure 5A), but also suggests that solubilization of the NR2 subunits from PSDs depends on the removal of spectrin. Noticeably, the presence of calcium ions did not affect the extractability of the NMDA-R NR1 subunit by Triton X-100, which is also consistent with the lack of calcium inhibition of the NR1–spectrin interaction *in vitro*. Overall, these observations suggest that spectrin has a role in stabilizing the NMDA-R complex and its linkage to synaptic membranes in a calcium-dependent manner.

Discussion

The NMDA-R and spectrin are both integral components of the PSD (Carlin *et al.*, 1983; Zagon *et al.*, 1986; Fifková and Morales, 1992; Malchiodi-Albedi *et al.*, 1993; Petralia *et al.*, 1994a,b; Kennedy, 1997; and see reviews by Fifková and Morales, 1992; Kennedy, 1997). The complexity of this structure has been established over the years and is now known to harbour a relatively large number of proteins including, in addition to NMDA-R and spectrin, CaMKII (Kennedy, 1997), PSD-95 (Kornau *et al.*, 1995; Niethammer *et al.*, 1996), SAP102 (Lau *et al.*, 1996; Müller *et al.*, 1996), chapsyn-110 (Kim *et al.*, 1996), fyn kinase (Suzuki and Okumura-Noji, 1995), PKA (Carr *et al.*, 1992), PKC (Suzuki *et al.*, 1993), α -actinin (Wyszynski *et al.*, 1997), actin, tubulin and a homologue of the neurofilament NF-L subunit (Walsh and Kuruc, 1992). The insolubility and electron-dense properties of PSDs probably originate from the existence of a dense mesh of interacting proteins, the nature of which is only

now beginning to emerge. In this context, the long cytoplasmic tails of the NMDA-R subunits have been established to interact directly with PSD-95 (Kornau *et al.*, 1995; Niethammer *et al.*, 1996), SAP102 (Lau *et al.*, 1996; Müller *et al.*, 1996), chapsyn-110 (Kim *et al.*, 1996), α -actinin-2 (Wyszynski *et al.*, 1997) and NF-L (Ehlers *et al.*, 1998).

The results of the present study demonstrate that spectrin and the NMDA-R subunits directly interact *in vitro* and probably also *in vivo*. The binding of spectrin is specific to the NMDA subtype since it does not exhibit any binding to the GluR1 subunit of the AMPA type (Figure 1). This finding supports the notion that the NMDA-R is anchored via spectrin to the actin cytoskeleton since spectrin is an actin-binding protein known to anchor various membrane proteins to the actin network (Bennett and Gilligan, 1993).

Recently, another actin-binding protein, α -actinin-2, was shown to interact with the NMDA-R subunits (Wyszynski *et al.*, 1997). Although belonging to the spectrin superfamily, α -actinin-2 binds to a region in NR2B distinct from spectrin (Figure 3C), suggesting that the two interactions are independent. Interestingly, both α -actinin-2 and spectrin interact with both NR1 and NR2B subunits (but spectrin also binds to NR2A), although the C-terminal domains of the latter do not share any significant sequence homology. NR2B seems to harbour several regions of interaction with spectrin, suggesting that the NR2B C-terminal domain and spectrin intertwine. This is in contrast to the interaction of NR2B with PSD-95 which takes place exclusively via the four C-terminal amino acids of NR2B (Kornau *et al.*, 1995; Niethammer *et al.*, 1996). Of relevance to the complex set of protein–protein interactions that take place in the PSD, both NR1 and spectrin bind to the neurofilament NF-L subunit via its rod domain (Frappier *et al.*, 1991; Ehlers *et al.*, 1998). The binding of both proteins to the same NF-L domain raises the possibility that spectrin may regulate the binding of NR1 to NF-L.

The interaction observed here between spectrin and the NMDA-R subunits was found to be inhibited by phosphorylation on the NR1 C-terminal domain (via PKC and PKA) and on the NR2B C-terminal domain (fyn) (Figure 4). The sensitivity of spectrin to the phosphorylation state of its substrates has been demonstrated already for the interaction of spectrin with other proteins (Lu *et al.*, 1985; Eder *et al.*, 1986; Iga *et al.*, 1997). Interestingly, while phosphorylation by PKC and PKA of NR1 causes a potentiation of the NMDA-R channel response (Chen and Huang, 1992; Raman *et al.*, 1996), application of exogenous fyn kinase to heterologously expressed NR1/NR2A enhances the channel activity while the activity of NR1/NR2B channels is not affected (Köhr and Seeburg, 1996). However, following LTP and taste learning, there is an increase in tyrosine phosphorylation of the NR2B subunit (Rosenblum *et al.*, 1996, 1997). This finding is in line with the impaired LTP and spatial learning observed in fyn mutant mice (Grant *et al.*, 1992). One may thus speculate that the dissociation of spectrin from a phosphorylated NR1 subunit contributes to the observed potentiation of the NMDA response while its dissociation from a tyrosine-phosphorylated NR2B subunit is part of the LTP-induced synaptic changes though it may

not have an overt effect on the activity of channels composed solely of NR1 and NR2B.

To add to the complexity, both calcium ions and CaM are found to inhibit the NMDA-R–spectrin interaction with effects displaying subunit specificity (Figure 5A and B). Both spectrin and NR1 are CaM-binding proteins (Carlin *et al.*, 1983; Ehlers *et al.*, 1996), and thus the observed inhibition by CaM of spectrin binding to NR1 can result from CaM binding to either one. This is in contrast to the case of α -actinin-2 where the inhibitory effect of CaM is due to competition on NR1 binding (Wyszynski *et al.*, 1997). The inhibition by calcium ions of spectrin binding to NR2B but not to NR1 (Figure 5A) cannot be attributed to a possible interaction of calcium ions with NR2B, as the latter does not possess an obvious calcium-binding motif, but rather with spectrin itself which harbours calcium-binding EF hands (Travé *et al.*, 1995). It is thus possible that in the native environment, spectrin dissociates from NR2B in the presence of calcium ions but not from NR1. This notion is supported by the calcium-dependent detachment of spectrin and NR2 subunits (but not of NR1) from synaptosomal membranes (Figure 7).

Recently, the calcium/CaM complex was shown to inactivate the NMDA-R channel via its direct binding to the NR1 cytoplasmic domain (Ehlers *et al.*, 1996). The NMDA-R also undergoes a calcium-dependent gradual loss of activity or ‘run-down’ which has been attributed to the calcium-induced detachment of a hypothetical regulatory protein linking the NMDA-Rs to the actin cytoskeleton and to calcium-dependent actin depolymerization (Rosenmund and Westbrook, 1993a,b). The present finding that calcium ions cause a significant loosening of both spectrin and NR2B from the synaptosomal membrane (Figure 7) suggests that spectrin, perhaps along with α -actinin-2, may well be the long sought linker protein. The direct interaction of the NMDA-R with spectrin may also account for the selective NMDA-R-induced spectrin breakdown that takes place in LTP and NMDA excitotoxicity (Lynch and Baudry, 1987; Siman and Noszek, 1988; Vanderklish *et al.*, 1995; Faddis *et al.*, 1997). Moreover, the observed extensive regulation of the NMDA-R–spectrin interactions by plasticity-related factors, such as PKC and fyn kinases, suggests a dynamic model in which the processes of synaptic activity and plasticity cause structural rearrangements of the proposed NMDA-R–spectrin–actin scaffold (Figure 8). It is interesting to point out that only the concerted action of calcium ions, CaM and phosphorylation would lead to the complete detachment of spectrin from the NMDA-R complex. The detachment, however, could be graded if it involves only one NMDA-R subunit. In any event, structural modifications of the NMDA-R–spectrin–actin scaffold may account for the mechanosensitivity of the NMDA-R channel activity and for the morphological changes that dendritic spines undergo following exposure to glutamate (Fifková and Morales, 1992; Papa and Segal, 1996).

Materials and methods

Plasmid construction and oligonucleotides

The cytoplasmic domains of GluR1 (DDBJ/EMBL/GenBank accession No. M36418) (residues 827–908), NR1a (DDBJ/EMBL/GenBank accession No. U08261) (residues 834–938), NR2A (DDBJ/EMBL/GenBank

Fig. 8. Schematic model of the putative dynamic linkage of the NMDA-R NR1 and NR2 subunits to actin through spectrin. (A) Under basal conditions, spectrin links the NMDA-R NR1 and NR2 subunits to the actin cytoskeleton. (B) Upon a rise in the intracellular calcium concentrations, spectrin binds calcium ions and detaches from the NR2 subunits. In addition, activated Ca^{2+}/CaM binds to either spectrin or NR1 (or to both), thus inhibiting spectrin binding to NR1. (C) Phosphorylation of NR1 by either PKC or PKA and of NR2B by fyn leads to a dissociation of spectrin from the phosphorylated subunits. N and C stand for the N- and C-termini, respectively.

accession No. M91561) (residues 838–1464) and NR2B (DDBJ/EMBL/GenBank accession No. M91562) (residues 1086–1481, 1086–1326, 1308–1481, 1086–1226 and 1204–1326) were amplified by PCR using the proof reading DNA polymerases Vent (New England BioLabs) or ExTaq (Takara). The PCR primers were as follows:

GluR1:

sense primer (5'-GGAATTCGAGTCTCTGCTACAAATC-3')

antisense primer (5'-GCTCTAGATCACAATCCTGTGGCTCC-3')

NR1a:

sense primer (5'-GAGATCGCCTACAAGCGAC-3')

antisense primer (5'-CCCAAGCTTAGCTCTCCCTATGACGGGA-3')

NR2A:

sense primer (5'-GGAATTCGAGACCTCTTCTACTGGAAG-3')

antisense primer (5'-GCTCTAGATCAAACATCAGATTCGATACTAGGC-3')

NR2B 1086–1481:

sense primer (5'-GGAATTCAGGACAGTCTAAAGAAG-3')

antisense primer (5'-GCTCTAGATCAGACATCAGACTCAATAC-3')

NR2B 1086–1326:

sense primer (5'-GGAATTCAGGACAGTCTAAAGAAG-3')

antisense primer (5'-GCTCTAGAGTCTTTCAGGCTCACGC-3')

NR2B 1308–1481:

sense primer (5'-GGAATTCGTGGACCTGCAGAAG-3')

antisense primer (5'-GCTCTAGATCAGACATCAGACTCAATAC-3')

NR2B 1086–1226:

sense primer (5'-GGAATTCAGGACAGTCTAAAGAAG-3')

antisense primer (5'-GCTCTAGATCAGGAGTAATTGTGCAGCT-3')

NR2B 1204–1481:

sense primer (5'-GGAATTCGTGGATTGGGAGGAC-3')

antisense primer (5'-GCTCTAGATCAGACATCAGACTCAATAC-3')

All clones were constructed in the *EcoRI*–*XbaI* sites in pmalC2 (New England BioLabs) except for NR1a C-terminal domain which was

constructed in *XmnI-HindIII* and called NR1 C-terminal domain. In addition to being inserted in the pmalC2 plasmid, NR1 (834–938) and NR2B (1086–1481) PCR products were blunt ligated to pGEX-3X plasmid (Pharmacia) in *SmaI-EcoRI* sites.

Expression and purification of recombinant proteins

The GluR1, NR1, NR2A and NR2B C-terminal domains were expressed in *Escherichia coli* strain UT5600 as MBP fusion proteins by transformation of the appropriate pmalC2 construct followed by induction with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 37°C for 2 h. The MBP- β -gal α -fragment was expressed directly from the pmalC2 plasmid. The grown bacteria culture was then harvested and lysed in ice-cold lysis buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA pH 7.4) by three repetitive freeze-thaw cycles followed by sonication. The lysed bacterial extract was centrifuged at 20 000 g for 20 min and the supernatant was used for the purification procedure. The MBP fusion proteins were then affinity purified on amylose resin (New England BioLabs) and eluted using 10 mM maltose (Sigma). On storage, purified MBP-NR1 and MBP-NR2B were found to be stable whereas the MBP-NR2A was not and underwent degradation. For this reason, most of the work was performed with MBP-NR1 and MBP-NR2B. The GST fusion proteins were expressed as above by transformation of the various pGEX-3X constructs. The grown bacterial culture was then harvested and lysed in ice-cold phosphate-buffered saline (PBS) as above. The lysed bacterial extract was centrifuged at 20 000 g for 20 min and the supernatant was incubated with glutathione resin (Pharmacia) for 2 h. The GST fusion proteins bound to the resin were washed extensively and used directly in the 'pull-out' experiments (see below).

Electrophoresis and immunoblotting procedures

Proteins were separated by either 8 or 6% SDS-PAGE (Laemmli, 1970). Immunoblotting was performed by using the ECL method according to the manufacturer's instructions (Amersham). The monoclonal anti-NR1 antibody (Chemicon) was used at a 1:500 dilution. The polyclonal anti-NR2A/B antibody (Sigma) was used at the final concentration of 0.25 μ g/ml. The polyclonal anti-spectrin antibody (230/235E, Chemicon) was used at a 1:500 dilution. The horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-mouse and goat anti-rabbit, Jackson) were used at a dilution of 1:5000 and 1:10 000, respectively.

The monoclonal anti-NR1 antibody recognizes an epitope between amino acids 660 and 811 of the NR1 subunit. The polyclonal anti-NR2A/B antibody was prepared using a peptide consisting of amino acids 1445–1464 of NR2A but it cross-reacts with NR2B as well. The polyclonal anti-spectrin antibody recognizes the erythrocyte isoform of brain spectrin (235/235E) which is present in dendrites and soma of neurons. According to the manufacturer, this antibody recognizes mainly the α -chain of spectrin.

Purification of brain spectrin

Brain spectrin was purified from bovine brain by low ionic extraction, ammonium sulfate precipitation and gel filtration as described (Davis and Bennett, 1983). The purified spectrin preparation shows after SDS-PAGE only two bands of $M_r = 230\,000$ and $235\,000$ visualized with silver and with anti-spectrin 230/235E antibody on Western blot (data not shown).

Iodination of spectrin

Purified brain spectrin (10 μ g) was iodinated by the chloramine T method using 1 mCi of 125 I. Mild iodination was achieved by incubating the protein with 5 mg/ml chloramine T for 20 s at 4°C before quenching the reaction with metabisulfite (5 mg/ml) and potassium iodide (5 mg/ml).

Filter overlay assays

Fusion proteins (5 μ g each) were fractionated by SDS-PAGE on 8% minigels, transferred to nitrocellulose filters and blocked for 1 h in overlay buffer [4% bovine serum albumin (BSA) in PBS] followed by an incubation with 1 μ g/ml 125 I-labelled brain spectrin in overlay buffer for 4 h at room temperature. Filters were washed in PBS and exposed to PhosphorImager (Molecular Dynamics).

Preparation of brain cytosolic extract

Brains from three mature male Lewis rats were removed immediately following decapitation. The brains were homogenized in 10% (w/v) ice-cold homogenization buffer [0.32 M sucrose, 5 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml aprotinin and 1 μ g/ml leupeptin pH 7.4]. The homogenate was centrifuged at 700 g for 10 min

and the supernatant recentrifuged at 20 000 g for 1 h. The resulting supernatant was saved for subsequent 'pull-out' experiments.

'Pull-out' assays

Rat brain cytosolic fraction (200 μ g of protein) was incubated batch-wise with GST fusion proteins (10 μ g) bound to glutathione-Sepharose beads (Pharmacia) at 4°C for 1 h. The beads were washed in PBS and bound proteins were eluted with SDS sample buffer (Laemmli, 1970) and detected by immunoblotting. When indicated, the brain extract was pre-boiled for 10 min to serve as control. It was centrifuged to clean up any aggregating material resulting from the boiling step. However, no pellet was observed, indicating that boiling did not cause aggregation.

Phosphorylation of fusion proteins

For NR1 phosphorylation by PKA and PKC, 5 μ g of purified fusion protein was incubated with 10 U of PKA (Calbiochem) or 0.01 U of PKC catalytic subunit for 30 min at 30°C. Phosphorylation reaction buffer contained 50 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM CaCl₂ and 50 μ M ATP. For labelling, 2 μ Ci of [γ - 32 P]ATP was included. For NR2B phosphorylation by fyn, 5 μ g of purified fusion protein was incubated with 10 U of fyn (Upstate Biotechnology) for 60 min at 30°C. Phosphorylation reaction buffer contained 100 mM Tris-HCl (pH 7.2), 125 mM MgCl₂, 25 mM MnCl₂, 2 mM EGTA, 0.25 mM Na₂VO₄ and 2 mM dithiothreitol (DTT). For NR2B phosphorylation by CaMKII, 5 μ g of purified fusion protein was incubated with 25 U of CaMKII (New England BioLabs) for 30 min at 30°C. Phosphorylation buffer contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM DTT, 0.1 mM EDTA, 100 μ M ATP, and (when applicable) 2.4 μ M CaM and 2 mM CaCl₂. For labelling, 2 μ Ci of [γ - 32 P]ATP was included.

Preparation of rat synaptosomes

Synaptosomes were prepared as described (Carlin *et al.*, 1980) with some modifications. Briefly, rat forebrains were homogenized (12–20 strokes at maximum speed) with a Teflon/glass homogenizer in 4 vols (w/v) of buffer A (0.32 M sucrose, 1 mM NaHCO₃, 10 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 100 μ M AEBSF). The homogenate was diluted further to 10% (w/v) and centrifuged at 700 g for 10 min. The supernatant was recentrifuged at 13 800 g for 10 min, and the pellet obtained was resuspended in 2.4 vols (w/v) of buffer A and layered on a sucrose density gradient. The gradient contained 6 ml of the resuspended pellet and 10 ml each of 0.85, 1.0 and 1.2 M sucrose solutions all containing 1 mM NaHCO₃. The gradient was run at 82 500 g for 2 h. The synaptosomal fraction migrated as a band between 1.0 and 1.2 M sucrose and was recovered.

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