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Rapid communication

Regulation of phosphorylation at Ser^{1303} of GluN2B receptor in the postsynaptic density

R. Prabhu Ramya, S. Suma Priya, M. Mayadevi, R.V. Omkumar *

Molecular Neurobiology Division, Rajiv Gandhi Centre for Biotechnology, Thycaud P.O., Thiruvananthapuram 695014, Kerala, India

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ABSTRACT

Neuronal *N*-methyl-D-aspartate subtype of ionotropic glutamate receptor (NMDAR) that plays essential roles in excitatory synaptic transmission is regulated by phosphorylation. However, the kinases and phosphatases involved in this regulation are not completely known. We show that the GluN2B subunit of NMDAR is phosphorylated at Ser^{1303} by protein kinase C (PKC) and is dephosphorylated by protein phosphatase 1 (PP1), but not protein phosphatase 2A (PP2A) in isolated postsynaptic density (PSD). Although PSD is known to harbor PKC, PP1 and PP2A, their ability to regulate phosphorylation of GluN2B-Ser^{1303} would depend on the accessibility of GluN2B-Ser^{1303} to these proteins. Since PSD preparation is likely to maintain the organization of its component proteins as inside neurons, accessibility of kinases and phosphatases to GluN2B-Ser^{1303} *in vivo* would be addressed by experiments using this system. Using an antibody specific for the phosphorylated state of GluN2B-Ser^{1303} we demonstrate that PP1 is the major phosphatase in rat brain PSD that can dephosphorylate the GluN2B-Ser^{1303} endogenous to PSD. We also show that PKC present in PSD can phosphorylate GluN2B-Ser^{1303}. The events reported here might be important in regulating GluN2B-Ser^{1303} phosphorylation *in vivo*.

1. Introduction

N-Methyl-D-aspartate (NMDA) receptors are ionotropic glutamate receptors which are critical for neuronal development and synaptic plasticity. NMDARs consist of the following classes of subunits, viz GluN1, GluN2, and GluN3, which form a heterotetrameric complex. There are 4 types of GluN2 subunits, GluN2 (A–D) of which GluN2A and GluN2B are found to be enriched in forebrain when compared to other regions (Ishii et al., 1992). In the adult rat, GluN2B subunit is expressed at its highest levels in the olfactory tubercle, hippocampus, olfactory bulb and cerebral cortex (Wang et al., 1995), all of which are principally part of forebrain.

NMDAR functions such as its channel properties or its intracellular trafficking are regulated by phosphorylation (Sanz-Clemente et al., in press) by a variety of protein kinases (Mammen et al., 1999; Roche et al., 1994) as well as phosphatases (Chen and Roche, 2007) resulting in changes in synaptic strength underlying many forms of synaptic plasticity (Lee, 2006). The GluN2B subunit of NMDAR has a phosphosite at Ser^{1303} which is phosphorylated *in vivo* (Omkumar et al., 1996). Ca^{2+}/CaM dependent protein kinase II (CaMKII) (Omkumar et al., 1996) as well as protein kinase C (PKC) (Liao et al., 2001) have been shown to phosphorylate this site *in vitro*. It is known that phosphorylation of GluN2B at this site by CaMKII inhibits Ca^{2+}/CaM independent binding of autophosphorylated CaMKII (Rajeevkumar et al., 2009) and promotes slow dissociation of preformed CaMKII–GluN2B complexes *in vitro* (Strack et al., 2000). A recent study shows that this binding and phosphorylation can be modulated by nucleotides like ATP which promotes Ser^{1303} phosphorylation (O'Leary et al., 2011). Although much is known about the phosphorylation of this site, regulation of dephosphorylation of this site by protein phosphatases remains relatively unexplored (Chen and Roche, 2007). Previous studies from our lab have shown the dephosphorylation of GluN2B-Ser^{1303} by phosphatases *in vitro* and suggested that this could serve as a potential regulatory mechanism (Rajeevkumar et al., 2009). Recent studies have also shown that repeated electroconvulsive shock, an effective therapy for depression patients, disrupts CaMKII–GluN2B binding by causing phosphorylation of the latter at Ser^{1303}, showing a modulation of glutamatergic transmission in depression like disorders and suggesting a reduction of GluN2B-Ser^{1303} phosphorylation in depression (Fumagalli et al., 2010). This emphasizes on the role of phosphatases in GluN2B function and the need for further investigation.

Abbreviations: NMDAR, *N*-methyl-D-aspartate receptor; PSD, postsynaptic density; CaMKII, calcium/calmodulin dependent protein kinase II; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; I-2, Inhibitor-2; I_1^{PP2A}, specific inhibitor of PP2A; OA, okadaic acid; CsA, Cyclosporin; BIM, bisindoylmaleimide; CyPA, Cyclophilin A; PhIC, phosphatase inhibitor cocktail.

* Corresponding author. Tel.: +91 471 2529483; fax: +91 471 2348096.
E-mail address: omkumar@rgcb.res.in (R.V. Omkumar).

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We have previously shown that purified preparations of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) can dephosphorylate Ser^{1303} in a GST fusion construct of the GluN2B fragment *in vitro* (Rajeevkumar et al., 2009). However, whether this site on GluN2B endogenous to PSD is accessible for dephosphorylation by PP1 and PP2A has not been investigated. Although significant amounts of PP1, PP2A and PP2B are present in PSD, their regulatory actions could be restricted due to the limited accessibility to phosphosites in the PSD as seen in the case of PSD-bound CaMKII which gets dephosphorylated by PP1, but not by PP2A (Strack et al., 1997).

The unique and complex structure of the PSD would dictate the mutual accessibility among these proteins and thus the occurrence of such regulatory mechanisms *in vivo*. PSD fraction isolated by detergent treatment of synaptosomes is known to have the same shape and size as the PSDs in intact tissue (Dosemeci and Reese, 1993; Vinade and Dosemeci, 2000). PSDs isolated from rodent brains are shown to be virtually identical to PSDs *in vivo* (Jordan et al., 2004). Isolated PSD is likely to maintain much of its *in vivo* organization and hence is considered to be a suitable structure for biochemical analysis of mutual interaction and accessibility among its constituent proteins (Dosemeci and Reese, 1993; Vinade and Dosemeci, 2000; Sheng and Hoogenraad, 2007).

The present paper reports the various kinases and phosphatases present in rat forebrain PSD which can influence the phosphorylation status of GluN2B-Ser^{1303} endogenous to PSD.

2. Materials and methods

2.1. Materials

ATP as disodium salt, Lambda phosphatase, Okadaic acid, Cyclosporin A, Cyclophilin A, etc. were from Sigma Chemicals, USA. Nitrocellulose membrane was from Pall Gelman, USA. Restriction and ligation enzymes and I-2 were from New England Biolabs, USA. Anti-phospho-GluN2B-Ser^{1303} was from Upstate (Millipore), USA. Bisindoylmaleimide and I_1^{PP2A} were from Calbiochem, Germany. Amersham ECL Plus western blot detection kit was from GE, USA. cDNAs for PP1 isoforms were obtained from Dr. Laura Trinkle-Mulcahy, University of Dundee, Scotland.

2.2. Preparation of PSD fractions from rat forebrain

The animals used in the preparation of PSD were albino Wistar male rats aged 40–45 days weighing an average of about 100 g. The animals were maintained at the animal house of Rajiv Gandhi Centre for Biotechnology and experiments conducted were in conformity with guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India and Institutional Animal Ethics Committee. The PSD fraction was prepared as described before (Rajeevkumar et al., 2009). Western blots of the PSD were done with various antibodies for the detection of protein phosphatases present.

2.3. Subcloning of PP1 isoforms to pGEX-6P-2 vector

The cDNA encoding PP1α was subcloned into the GST containing vector pGEX-6P-2 by directional cloning. The vector pGEX-6P-2 as well as the vector pECFP-C1 containing the PP1α isoform were double digested with the restriction endonucleases EcoR1 and Sal1. This released the PP1α cDNA insert from the latter. The cDNAs of PP1β and PP1γ were amplified by PCR using the pECFP-C1 template with the respective primers at 5′ and 3′ ends containing the restriction sites BamH1 and Xho1. The PP1 isoforms were ligated to pGEX-6P-2 vector. The ligated constructs were subjected to expression in BL21(DE3)pLys strain of *Escherichia coli*.

2.4. Expression of GST fusion proteins

The fusion proteins having GluN2B sequence (GluN2B amino acid residues 1271–1311), PP1α, PP1β and PP1γ (about 330 amino acids each) fused to GST were expressed using prokaryotic expression vectors pGEX-2T-GluN2B (Praseeda et al., 2004) and pGEX-6P-2 PP1α/β/γ. For expression of the protein, the construct was transformed into the *E. coli* strain BL21(DE3)pLys. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM in the case of GST-GluN2B (Rajeevkumar et al., 2009) and 0.2 mM for GST-PP1 isoforms. Preparation of crude lysates with the expressed proteins was conducted as described before (Rajeevkumar et al., 2009).

2.5. Purification of GST-fusion proteins

The crude lysates containing the GST-PP1 isoforms were purified. For this purpose, glutathione Sepharose beads were used. Glutathione Sepharose 4B beads (50 μl) were washed thoroughly with PBS and were mixed separately with each of the GST-fusion proteins and were subjected to incubation for 1 h at 4 °C with agitation. After this, the beads were again washed with 1X PBS to remove unbound proteins if any. Subsequently, about 500 μL of elution buffer (10 mM glutathione in 50 mM Tris, pH 8.0) was added and the mix was incubated at 4 °C with agitation for 30 min. The supernatant was collected and was subjected to concentration using Amicon ultra filter concentrators (Millipore) with molecular weight cut off value of 30 kDa. The purified protein thus made was kept at −80 °C for long term storage.

2.6. Dephosphorylation of phospho-GST-GluN2B by purified phosphatases in vitro

GST-GluN2B was phosphorylated by α-CaMKII *in vitro* using 300 μM ATP at 30 °C for 10 min. The reaction mixture also consisted of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 0.4 mM EGTA, 1.3 mM $CaCl_2$, 17 μM CaM and 0.2 mg/ml BSA. The phosphorylation was stopped using 5 μM staurosporine, a general kinase inhibitor. The phospho-GST-GluN2B-Ser^{1303} (2 μg) was incubated with about 25 μg of purified phosphatase either with or without phosphatase inhibitor cocktail (PhIC: 50 mM sodium fluoride, 5 mM sodium orthovandate, 5 mM EDTA and 50 mM β-glycerophosphate) at 30 °C for about 45–60 min. PhIC preincubation with the purified phosphatases was for about 15–20 min in ice. Samples were subjected to western blotting (Towbin et al., 1979) followed by chemiluminescence based detection using ECL kit from GE. The primary antibody used was rabbit anti-human phospho-GluN2B-Ser^{1303} and the secondary antibody was anti-rabbit IgG conjugated to horse radish peroxidase. The substrate for chemiluminescence was used for generating the signal and the blot was subjected to autoradiography using an X-ray film. The exposure time for autoradiography was usually 2–3 min.

2.7. Phosphorylation status of GluN2B endogenous to PSD

The phosphorylation of GluN2B endogenous to PSD by endogenous kinases was carried out by providing ATP at a concentration of 50 μM to PSD in presence of 1.3 mM $CaCl_2$ in 50 mM Tris buffer (pH 8.0) for 15 min. To inhibit PKC activity, 25 μM of PKC specific inhibitor, bisindoylmaleimide (BIM) was used. Initially PSD was subjected to treatment with 3 U/μL of lambda phosphatase for 30 min to completely dephosphorylate phospho-GluN2B-Ser^{1303}. After incubation, PhIC was added to stop the phosphatase activity,