ACCELERATED COMMUNICATION

Evidence for Direct Protein Kinase-C Mediated Modulation of N-Methyl-D-aspartate Receptor Current

GUEY-YING LIAO, DAVID A. WAGNER,1 MICHAEL H. HSU, and JOHN P. LEONARD
Laboratory of Integrative Neuroscience (G.-Y.L., D.A.W., M.H.H., J.P.L.) and Laboratory for Molecular Biology (D.A.W., J.P.L.), Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois
Received October 11, 2000; accepted February 2, 2001
This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT
Protein kinase-C (PKC) activation differentially affects currents from N-methyl-D-aspartate (NMDA) type glutamate receptors depending upon their subunit composition. Experiments using chimeras initially indicated that the cytoplasmic C-terminal tails of NR2B (responsive to PKC) and NR2C (unresponsive to PKC) subunits contain the amino acid residues responsible for the observed disparity of PKC effects. However, truncation and point mutation experiments have suggested that PKC action on NMDA receptors may be entirely indirect, working via the phosphorylation of associated proteins. Here we suggest that PKC does, in fact, affect NR2B/NR1–011 NMDA currents by direct phosphorylation of the NR2B tail at residues S1303 and S1323. Replacement of either of these residues with Ala severely reduces PKC potentiation. To verify that S1303 and S1323 are sites of direct phosphorylation by PKC, synthetic peptides from the regions surrounding these sites were used as substrates for in vitro assays with purified rat brain PKC. These results indicate that PKC can directly phosphorylate S1303 and S1323 in the NR2B C terminus, leading to enhanced currents through NMDA receptor channels. The direct action of PKC on certain NMDA receptor subtypes may be important in any physiological or pathological process where PKC and NR2B/NR1 receptors interact.

N-Methyl-D-aspartate (NMDA) receptors are a calcium-permeant, depolarization-dependent subtype of ionotropic glutamate receptors that mediates a wide variety of physiological and pathological processes in the central nervous system, including development of proper neuronal circuits (Constantine-Paton et al., 1990), certain learning tasks (Morris et al., 1986; Sakimura et al., 1995), and forms of synaptic modification (Collingridge and Bliss, 1995; Tsien et al., 1996; Ito et al., 1997) and excitotoxicity (Choi, 1988). NMDA receptors are oligomeric complexes generally composed of two types of subunit, the coagonist glycine binding subunit NR1 and the glutamate-binding subunits NR2A–D (Hollmann and Heinemann, 1994). The four NR2 subunits have large cytoplasmic carboxyl termini and are distinct in their functional properties, regulation, and temporal and spatial expression (Hollmann and Heinemann, 1994; Monyer et al., 1994).

Protein phosphorylation regulates multiple properties of ligand-gated ion channels, including clustering, desensitization, and peak currents (Swope et al., 1999). Within the NMDA subfamily of glutamate receptors, modulation of peak currents by PKC depends primarily on the NR2 subunits expressed (Kutsuwada et al., 1992). Enhancement by PKC is pronounced for receptors containing the NR2A or NR2B subunits, but absent for receptors containing the NR2C or NR2D subunits (Mori et al., 1993; Wagner and Leonard, 1996). Alternatively spliced variants of the NR1 subunit have more subtle effects on the level of PKC potentiation of currents (Logan et al., 1999). Experiments using chimeras between the NR2B (responsive to PKC) and NR2C (unresponsive to PKC) subunits indicate that the C-terminal region of the NR2B subunit is the critical structural domain for PKC-mediated current potentiation (Mori et al., 1993). However, whether the up-regulation of current response is mediated by

This work was supported by Grant R01-NS31962–02 from the National Institutes of Health (J.P.L.).

1 Current Address: Department of Physiology, University of Wisconsin, Madison, WI 53706.

ABBREVIATIONS: NMDA, N-methyl-D-aspartate; PKC, protein kinase C; kb, kilobase pairs; MOPS, (3-[N-morpholino]propanesulfonic acid); BOS, barium oocyte solution; PDBu, phorbol ester dibutyrate; aa, amino acid(s); PSD-95, postsynaptic density protein 95; CaMK, calmodulin-dependent kinase.
PKC phosphorylation of the NR2B C terminus has remained unclear. Although biochemical study has shown that NR2A and NR2B are phosphorylated by PKC (Leonard and Hell, 1997), no specific sites have been defined. Here we report the identification of two important serine residues, S1303 and S1323, in the NR2B C-terminal region that are directly phosphorylated by PKC and control the PKC-mediated enhancement of NR2B/NR1 receptor currents.

Materials and Methods

Plasmid Construction. Chimeras 1 and 2 were C-terminal chimeras constructed by exchanging the Clal–NcoI fragment of pBKSA2 and pBKSA3, which encodes the mouse NR2B and NR2C subunits, respectively, as described in Mori et al. (1993). Chimeras 3 to 5 were three mutant NR2B subunits containing different regions of the NR2B C terminus and part of the NR2C C terminus (Fig. 1). The fragments used to construct these chimeras were, for chimera 3, the 3.8-kb Clal-Eco47III fragment of NR2B and the 4.0-kb Eco47III-ClaI fragment of NR2C; for chimera 4, the 4.1-kb ClaI-Stul fragment of NR2B and the 4.0-kb Etel-ClaI fragment of NR2C; and, for chimera 5, the 6.4-kb SpeI-Ehel fragment of chimera 1 and the 1.3-kb StuI-SpeI fragment of NR2B. Deletions 1 and 2 were in-frame, C-terminal deletion mutants. Deletion 1 was constructed by deleting a 1008-base-pair Bpu1102I fragment. Deletion 2 was made by deleting a 214-base-pair EcoRI fragment followed by a fill-in. Site-specific mutagens S1303A, S1323A, S1354A, the double and triple mutants, were constructed by polymerase chain reaction mutagenesis using Phu polymerase and confirmed by dideoxynucleotide sequencing.

RNA Preparation and Injection. Plasmids pBKSA Clal–EcoRI, Clal–SpeI containing cDNAs encoding mouse NRAA and NR2B subunits, respectively, were kindly provided by Dr. Masayoshi Mishina (University of Tokyo, Japan). Complementary RNAs were transcribed in vitro using T3 RNA polymerase with linearized templates. Xenopus laevis oocytes were prepared as described previously (Liao and Leonard, 1999) and injected with 10 ng of RNAs in a 1:1 M ratio of NR1 to NR2 wild-type or mutant constructs.

Electrophysiological Recordings. All experiments were performed in Ca2+- and Mg2+-free solutions to avoid the contribution of endogenous Ca2+- and Mg2+-dependent Cl− channels to the NMDA response (Leonard and Kelso, 1990) and the voltage-dependent Mg2+ block of NR2A receptors. The standard recording solution, barium oocyte solution (BOS), contained 96 mM NaCl, 2 mM KCl, 5 mM HEPES, pH 7.5, and 2.8 mM CaCl2. Agonist-containing solution had 100 μM NMDA and 10 μM glycine in BOS. Phorbol-12,13-dibutyrate (PDBu) was prepared as a 1 mM stock in dimethyl sulfoxide. Currents were recorded using a 2-electrode, voltage-clamp technique. Current response was evoked by bath perfusion of agonist solution at 5 ml/min for 12 s (bath = 200 μl) followed by a wash-out with BOS in a 40-s recording while the oocyte membrane potential was held at −80 mV. Currents recorded before PDBu (20 nM) or vehicle (BOS) treatment for 10 min were normalized to 100% as baseline. Error bars indicate SEM, and significance was assessed using a Student’s t test throughout.

PKC Phosphorylation of Synthetic Peptides. Peptides were synthesized by the Protein Research Laboratory of University of Illinois at Chicago. For in vitro phosphorylation by PKC, synthetic peptides (5 μM) were phosphorylated for 10 min at 30°C in a solution containing 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM Na3VO4, 1 mM diethiothreitol, and 15 mM MgCl2, along with 100 μg/μl phosphatidyl serine, 10 μg/μl diacylglycerol, 100 μM ATP, 25 ng of purified rat brain PKC (α1, β1, β2, and γ isoforms), and 10 μCi (~300 Ci/mmol) [γ-32P]ATP as described (PKC assay kit; Upstate Biotechnology, Lake Placid, NY). The reaction mixture was then transferred to a filter paper followed by three washes with 0.75% phosphoric acid and one with acetone. Radioactivity was determined by liquid scintillation counting.

Results

Chimeras and Deletions Narrowed Down the Target Region. Using a series of chimeric subunits constructed between the NR2B (responsive to PKC) and NR2C (unresponsive to PKC) subunits and two NR2B C-terminal truncation mutants, a 127-aa region (residues 1245–1371) within the NR2B C terminus was first found to be important for PKC modulation. Chimeras 1 and 2 of Fig. 1 confirm earlier work (Mori et al., 1993), in which the NR2C C-terminal tail was exchanged for the NR2B C terminus conferring PKC sensitivity onto the normally insensitive NR2C/NR1 receptors. Chimeras 3 and 4 show that neither the first 284 aa (839–1122) nor the first 406 aa (839–1244) of the NR2B C-terminal tail are adequate to support PKC potentiation. In contrast, Chimera 5 shows that the last 238 aa (1245–1482) of the C terminus are able to support PKC potentiation. Two truncation mutants were then made by deleting part of the last 238 aa in an attempt to further narrow down the critical region for PKC modulation. Deletions 1 and 2 had residues 1036 to 1371 and 1240 to 1310 deleted, respectively, which includes the first 127 aa and the first 66 aa of 1245 to 1482, respectively. As shown in Fig. 1, deletions 1 and 2 result in a total loss and a significant decrease in PKC potentiation, respectively. Comparison between results from chimeras and from deletions 1 and 2 of NR2B, narrowed down the critical region from the entire C terminus (644 aa) to the last 238 aa and then to residues 1245 to 1371 (127 aa). Vehicle controls for the receptors listed in Fig. 1 are −5 ± 4% (n = 14), −1 ± 3% (n = 6), 5 ± 2% (n = 7), 13 ± 10% (n = 7), 13 ± 11% (n = 5), 2 ± 4% (n = 5), −7 ± 1% (n = 6), 9 ± 7% (n = 12), and −3 ± 4% (n = 9) for NR2B, NR2C, chimeras 1 to 5, and deletions 1 and 2, respectively.

Identification of Two Serine PKC Sites Controlling Current Modulation. Within this critical region, three potential serine PKC sites (Kennelly and Krebs, 1991), S1303, S1323 and S1354, were mutated to alanine by primer-mis-
match polymerase chain reaction. Threonines in NR2B are not sites of PKC phosphorylation (Hall and Soderling, 1997). Figure 2 summarizes evidence of direct PKC modulation of currents through NR2B/NR1 receptors. Figure 2b shows representative current traces from wild-type and Ser-to-Ala point-mutated NR2B/NR1 receptors. The expression of receptor protein was apparently completely normal as estimated by the size of whole-cell currents (~0.4 pA) for all mutants and wild-type. Activation of PKC by PDBu potentiated currents from wild-type receptors by 201 ± 16% (n = 18), whereas potentiation of S1303A was reduced to only 57 ± 8% (n = 16) and that of S1323A to only 47 ± 8% (n = 16) of baseline. The third candidate site, S1354A, showed no significant change in PKC potentiation from wild-type (238 ± 19%, n = 18). The level of current potentiation found with S1303A is roughly the same as that from deletion 2 of Fig. 1, which deletes 71 residues including S1303. Potentiation of currents from double mutant S1303A/S1323A, S1303A/S1354A, and S1323A/S1354A were potentiated as follows: 30 ± 8%, 59 ± 15%, and 50 ± 14%; n = 20, 10, and 11 cells, respectively. Potentiation of the double mutant S1303A/S1323A was reduced significantly compared with S1303A but not S1323A. Double mutants, which included S1354A, were not significantly different from their respective single mutants (S1303A and S1323A). The potentiation for the triple mutant, S1303A/S1323A/S1354A, was 39 ± 10% (n = 16), which was not significantly changed from the double mutant S1303A/S1323A. Thus, mutation of S1354 is unimportant for PKC potentiation. Vehicle controls for tested receptors (from wild-type to triple mutant in Fig. 2c) are 0 ± 5% (n = 8), -7 ± 4% (n = 7), 4 ± 4% (n = 7), -6 ± 2% (n = 7), -2 ± 4% (n = 9), -10 ± 4% (n = 5), -9 ± 3% (n = 5), and -5 ± 7% (n = 6) respectively. These data are the first to demonstrate that mutation of serine residues of an NR2 subunit affects PKC potentiation of NMDA receptor currents.

**S1303 and S1323 Are Good Substrates for PKC In Vitro.** Because there may be other reasons that any particular amino acid residue is required for modulation of a receptor, we sought direct evidence that S1303 and S1323 are good substrates for phosphorylation by purified rat brain PKC (Fig. 3). When synthetic peptides, 11 residues in length, constructed around S1303 (1298–1308) and S1323 (1318–1328), were incubated with purified rat brain PKC and [γ-32P]ATP, they incorporated 32P at a level within 15 to 17% of that of the myelin basic protein control peptide (38.2 ± 3.6 μmol/min/mg). S1303 and S1323 incorporated 32P at the level of 5.6 ± 0.8 and 6.6 ± 1.3 μmol/min/mg, respectively (n = 5). Identical peptides mutated at S1303A and S1323A were not substrates for PKC, the levels of 32P incorporation were 0.19 ± 0.04 and 0.30 ± 0.08 μmol/min/mg, respectively (n = 5). Neither wild-type S1354 nor S1354A peptides are PKC substrates, 0.01 ± 3.3 × 10⁻³ and 0.02 ± 0.01 μmol/min/mg, respectively (n = 3). These results suggest that S1303 and S1323 directly mediate PKC potentiation via their ability to be phosphorylated by PKC.

**Discussion**

Taking the electrophysiological and biochemical data together, we clearly demonstrate that the majority of PKC potentiation of NR2B/NR1 receptor currents in oocytes is mediated by direct phosphorylation of specific serine residues, S1303 and S1323, within the NR2B C terminus. Two-dimensional phosphopeptide mapping has shown that major phosphorylation of NR2B by a mixture of PKC isoforms from rat brain (α, β, and γ) occurs at more than one site and that these differ from sites of PKA phosphorylation (Leonard and Bell, 1997). S1303 and S1323 would be predicted to represent two of the spots on the two-dimensional gels. Back-phosphorylation of NR2B, immunoisolated from rapidly collected and homogenized rat brain in the absence and presence of phosphatase inhibitors, showed that it is phosphorylated in vivo at those sites recognized by PKC in the conventional two-dimensional phosphopeptide mapping (Leonard and Hell, 1997). The phosphorylation stoichiometry of NR2B also suggests that it is an efficient substrate for PKC and may be phosphorylatable under normal physiological conditions (Leonard and Hell, 1997). The present results using deletions of NR2B and chimeric constructs of NR2B and NR2C have refined the domain that is sufficient to confer PKC sensitivity onto NR2C. How small a domain from NR2B will suffice remains in question. The critical serines, S1303 and S1323, are only 20 residues apart, so perhaps a domain as small as 24 to 30 residues, including S1303 and S1323, would suffice to confer PKC sensitivity onto NR2C and possibly even onto NR2D.

The finding of direct modulation of NMDA currents by PKC may be somewhat surprising given the presence of substantial indirect pathways active in some neurons and in oocytes injected with whole-brain mRNA (Lu et al., 1999). In neurons isolated from hippocampal slices, PKC acts indirectly via activation of Src, although Src is not thought to be directly activated by PKC, but rather via yet another intermediary, CAKβ/PYK2 (Lu et al., 1999). Perhaps such an indirect mechanism explains the small residual PKC potentiation found on expression of the double mutant S1303A/S1323A in oocytes. However, because the present results demonstrate a direct action of the typical family of rat brain PKC isoforms on two sites in the NR2B C terminus that affect current potentiation, it is likely that at least part of the physiological modulation by PKC occurs by direct phosphor-
ylation of these two sites. Even so, the percentage of PKC-mediated current potentiation for NMDA receptors is quite different in oocytes than in cultured hippocampal neurons. For oocytes injected with NMDA receptor subunits or whole-brain mRNA, the degree of potentiation usually is more than 2-fold (Kelso et al., 1992; Mori et al., 1993; Wagner and Leonard, 1996; Lu et al., 1999), whereas in cultured hippocampal neurons, the observed potentiation is usually less than 50% (Lu et al., 1999). Recently, the postsynaptic density protein 95 (PSD-95), an NMDA receptor-associated protein, was found to produce an inhibitory effect on PKC potentiation of NMDA currents when coexpressed with NR2A/NR1 or NR2B/NR1 receptors in oocytes (Yamada et al., 1999; Liao et al., 2000). The inhibition of PSD-95 seems to be dependent on the amount of PSD-95 coinjected (Yamada et al., 1999). Whether the difference in degree of direct PKC potentiation is caused by the absence versus presence of PSD-95 in oocytes versus in neurons and whether PSD-95 might normally play a negative role in the direct PKC phosphorylation of NR2B subunit remain to be investigated.

It is noteworthy that the stoichiometry of binding between PSD-95 and NMDA receptors may vary during development and in certain pathological conditions. For example, during early developmental stages, NR2B is primarily associated with another PSD-95 family protein, SAP102, and gradually with PSD-95, which is expressed in later developmental stages (Sans et al., 2000). Whether SAP102 also plays a role in PKC potentiation for NR2B/NR1, the dominant subtype of NMDA receptors for young animals, is currently unknown. During transient global ischemia, a significant decrease in the association of PSD-95 with NR2A and NR2B subunits has been shown (Takagi et al., 2000). In addition, a marked translocation of CaMK II and PKC-β to postsynaptic densities were induced by transient cerebral ischemia (Hu et al., 2000). This raises the possibility that NMDA receptor activity may be up-regulated by the translocated kinases, which could have an important consequence in NMDA receptor-mediated excitotoxicity.

Evidence from total deletion of the C-terminal region of NR2A has led to the conclusion that PKC potentiation of NMDA currents in oocytes is independent of the presence of the C terminus (Zheng et al., 1999). This conclusion from results using NR2A is in contrast to the present results using PKC site mutants and chimeras of NR2B and to previous results from NR2B chimeras (Mori et al., 1993). These results are also at odds with similar truncation experiments on NR2A performed using human embryonic kidney 293 cells in which the PKC potentiation of glutamate-stimulated rise in intracellular calcium depends on a 139-aa region (residues 1267–1406) of the NR2A C terminus (Grant et al., 1998). This region contains a stretch of 77 aa (1267–1343) that shares 64% sequence homology with a similar region within NR2B (1281–1355) and also contains sites homologous to S1303 and S1323 of NR2B (S1291 and S1312 in NR2A). For this reason, it is possible that the homologous serines in NR2A will also mediate PKC potentiation of NMDA currents. The previous putative PKC site Ser-to-Ala replacement studies that also led to the conclusion that PKC modulation was not via direct phosphorylation of the NMDA receptor were performed on sites outside of the C-terminal region because of the mistaken assumption that glutamate receptor topology would match that of the nicotinic acetylcholine receptor and have extracellular C termini (Sigel et al., 1994). PKC sites S1303 and S1323 could account for most of the discrepancy between the PKC-sensitivity of NR2A or NR2B-containing receptors and the PKC-insensitivity of NR2C and NR2D-containing receptors. This is because sites homologous to S1303 and S1323 of NR2B are clearly present in NR2A (S1291 and S1312), but clearly absent from NR2C and NR2D. Alignment of NR2C with NR2B shows that either the serine itself (for site aligned to 1303) or the upstream arginine (for the site aligned to 1323) is missing in the NR2C C terminus. These sites in NR2B are best aligned with a gap region in NR2D. Extensive mutagenesis of putative PKC sites has also been done on NR1 (Yamakura et al., 1993), demonstrating that PKC sites in NR1 are not required for PKC potentiation of currents. Because injection of NR1 cRNA alone into oocytes yields small currents that are nevertheless potentiated by PKC activation (Logan et al., 1999), the possibility of direct PKC action on the endogenous glutamate receptor subunit XenU1 must be considered (Soloviev and Barnard, 1997). There are no sites in the short C terminus of XenU1 that are at all homologous to S1303 or S1323 and only one possible PKC site (Ishimaru et al., 1996). In addition, any potentiating effect of PKC activation on NR1/XenU1 currents would be insufficiently large to account for more than a small fraction of the residual PKC potentiation in the NR2A double mutant.

The mechanism whereby phosphorylation of S1303 and S1323 leads to an increase in NMDA current remains unknown. There does not seem to be a change in the sensitivity to inhibition by Mg\(^{2+}\) either in recombinant receptors expressed in oocytes (Wagner and Leonard, 1996) or in neurons (Xiong et al., 1998). There is evidence from single channel recordings in human embryonic kidney 293 cells that an

| S1303 | LRRGHSYDTFV |
| S1303A | LRRGHAYDTFV |
| S1323 | APRSV$LDKKG |
| S1323A | APR$VALDKKG |
| S1354 | FANKS$VTAG |
| S1354A | FANKSAVTAG |

**Fig. 3.** Purified rat brain PKC phosphorylates S1303 and S1323 but not S1354 in vitro. The synthetic peptides indicated were incubated with rat brain PKC and [\(\gamma\]32P]ATP to measure incorporation of 32P. Point mutants S1303A and S1323A showed significantly reduced 32P incorporation compared with their wild-type controls (S1303 and S1323) \(**p < 0.01\). No significant levels of 32P were incorporated into either S1354 or S1354A.
increase in probability of opening is the primary effect with no change in unitary conductance (Xiong et al., 1998). Based on open channel block by MK801, there is also evidence that an increase in the total number of NMDA receptors may underlie PKC potentiation in oocytes (Zeng et al., 1999). The previously noted requirement for an intact actin cytoskeleton in PKC potentiation (Wagner and Leonard, 1999) may suggest that actin is involved in translocation of new receptors to the surface.

NR2A and NR2B are substrates for PKC and the same pattern of phosphorylation of NR2B seen in vitro was found in vivo, suggesting that direct PKC action on native NMDA receptors may modulate currents at synapses (Leonard and Hell, 1997). The present identification of two C-terminal PKC phosphorylation sites that are required for substantial PKC-mediated potentiation of NMDA currents also supports a direct modulatory action of PKC on NMDA receptors. In addition, S1303 had previously been identified as a major site of CaMKII phosphorylation in NR2B (Omkumar et al., 1996). Because disruption of either PKC or CaMKII activity can prevent induction of LTP (Malinow et al., 1989), it is conceivable that a concerted activation of these two Ca\(^{2+}\)-dependent kinases by calcium, entering via the NMDA receptor channel, leads to a positive feedback necessary for LTP induction.

Acknowledgments

We thank Dr. Masayoshi Mishina for the cDNA clones of mouse NMDA receptor subunits, Dr. Terry P. Snutch for suggesting the use of synthetic peptides, Dr. Stephen R. Kelso for helpful comments on the manuscript, and Richard Koche for participation in part of this study.

References


Send reprint requests to: Dr. J. P. Leonard, Dept. Biological Sciences, MC/067, University of Illinois at Chicago, 840 W. Taylor St., Chicago, IL 60607. E-mail: leonard@uic.edu