Protein kinase C potentiation of *N*-methyl-D-aspartate receptor activity is not mediated by phosphorylation of *N*-methyl-D-aspartate receptor subunits

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***N*-methyl-D-aspartate receptors (NMDARs) are Ca^{2+}-permeable glutamate-gated ion channels whose physiological properties in neurons are modulated by protein kinase C (PKC). The present study was undertaken to determine the role in PKC-induced potentiation of the NR1 and NR2A C-terminal tails, which serve as targets of PKC phosphorylation [Tingley, W. G., Ehlers, M. D., Kameyama, K., Doherty, C., Ptak, J. B., Riley, C. T. & Huganir, R. L. (1997) *J. Biol. Chem.* 272, 5157–5166]. Serine residue 890 in the C1 cassette is a primary target of PKC phosphorylation and a critical residue in receptor clustering at the membrane. We report herein that the presence of the C1 cassette reduces PKC potentiation and that mutation of Ser-890 significantly restores PKC potentiation. Splicing out or deletion of other C-terminal cassettes singly or in combination had little or no effect on PKC potentiation. Moreover, experiments involving truncation mutants reveal the unexpected finding that NMDARs assembled from subunits lacking all known sites of PKC phosphorylation can show PKC potentiation. These results indicate that PKC-induced potentiation of NMDAR activity does not occur by direct phosphorylation of the receptor protein but rather of associated targeting, anchoring, or signaling protein(s). PKC potentiation of NMDAR function is likely to be an important mode of NMDAR regulation *in vivo* and may play a role in NMDA-dependent long-term potentiation.**

excitatory amino acids | site-directed mutagenesis

N-methyl-D-aspartate receptors (NMDARs) mediate slow excitatory transmission in the brain and are thought to play a role in synaptogenesis, formation of neuronal circuitry, synaptic plasticity, and learning and memory (for review, see refs. 1 and 2). NMDARs in mammalian brain are encoded by a gene family comprised of the NR1 and NR2A-D genes (3). Alternative splicing of NR1 mRNA provides further molecular diversity of NMDARs (for review see ref. 4). Altogether, eight receptor splice variants are possible, at least seven of which are expressed in the central nervous system. The variants arise through the insertion or deletion of three short exon cassettes in the N-terminal (N1) and C-terminal (C1 and C2) domains. Splicing out of the exon segment encoding C2 removes a stop codon, resulting in an ORF that encodes an unrelated sequence C2′ at the distal end of the C terminus. NR1 splice variants differ in their patterns of temporal and spatial expression (5, 6), their pharmacological properties (7–12), their ability to be phosphorylated by protein kinases (13, 14), their efficiency of insertion at the cell surface (15), and their subcellular localization in heterologous expression systems (16).

Target sites of kinase-induced phosphorylation have been identified on the NR1, NR2A, and NR2B subunits (13, 14, 17). Protein kinase C (PKC) phosphorylates serine residues 889, 890, and 896 within the C1 exon of the NR1 subunit; protein kinase A (PKA) phosphorylates serine residue 897 (14). The relation of PKC-induced phosphorylation of the NR1 and NR2 subunits to PKC-induced potentiation of NMDA channel activity is, however, unclear.

A number of intracellular neuronal proteins, including members of the *p*ostsynaptic *d*ensity 95 (PSD-95/SAP90) subfamily of membrane-associated putative guanylate kinases, α-actinin, calmodulin, neurofilament protein NF-L, and yotiao bind via the C tails of NMDAR to mediate membrane targeting, attachment to the neuronal cytoskeleton, and stabilization of NMDARs (18–26). PKC-induced phosphorylation of Ser-890 within the C1 cassette disrupts one or more of these interactions and disperses surface-associated clusters of NR1 subunits (14).

PKC is likely to be an important regulator of neuronal NMDARs *in vivo.* The PKC-activating phorbol ester, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), enhances NMDA-elicited currents in trigeminal dorsal horn and hippocampal neurons in slice (27, 28) and in isolated hippocampal neurons in culture (29). TPA potentiates recombinant NMDARs expressed in *Xenopus* oocytes (7, 8, 30–32) and HEK-293 cells (33). Activation of G protein-coupled receptors, including phosphoinositol-coupled metabotropic glutamate receptors (34, 35), μ opioid receptors (36), and muscarinic receptors (37), potentiates NMDARs via activation of PKC.

The present study was undertaken to examine the role in PKC-induced potentiation of the NR1 and NR2A C-terminal tails, known to be targets of PKC phosphorylation. Our study shows that splicing in of the C1 cassette, which contains all known sites of phosphorylation on the NR1 subunit, reduces PKC potentiation (see refs. 8, 49, and 50). Mutation of Ser-890, implicated in receptor clustering (16), partially restores potentiation. Experiments involving truncation mutants reveal the unexpected finding that NMDARs assembled from subunits lacking all known sites of PKC phosphorylation show marked PKC potentiation. These results indicate that PKC-induced potentiation of NMDAR function does not occur by direct phosphorylation of the receptor but rather of associated protein(s) to increase open probability and/or insertion of new channels into the plasma membrane.

Materials and Methods

Nomenclature. Splice variants of the NR1 subunit are denoted by subscripts indicating presence (1) or absence (0) of alternatively spliced exon cassettes (N1, C1, and C2). $NR1_{011}$ (also termed NR1A or NR1-1a) lacks the N1 cassette but contains the C1 and C2 cassettes; $NR1_{100}$ (NR1G or NR1-4a) contains N1 but lacks C1 and C2 (4). Subunits lacking C2 contain an unrelated sequence C2′. The 33-amino acid sequence between the fourth transmembrane domain (M4) and first C-terminal splice site, C0, is common to all NR1 variants. Residues in the C-terminal

Abbreviations: NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; PKC and PKA, protein kinase C and A; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; M4, fourth transmembrane domain.

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domain were assigned numbers according to their position in $NR1_{011}$.

Expression Constructs. Rat $NR1_{011}$, $NR1_{010}$, $NR1_{001}$, and mouse $\varepsilon 1$ (rat NR2A homolog) cDNAs were gifts from S. Nakanishi (Kyoto University, Kyoto), V. R. Anantharam (University of Massachusetts, Worcester, MA), and M. Mishina (Niigata University, Niigata, Japan). Other receptor cDNAs were cloned or engineered (7). cDNAs were linearized with *Bam*HI (wild-type and mutant $NR1_{100}$ and $NR1_{111}$) or *Not*I (other NR1s and NR2A), and capped mRNAs were synthesized as described (Ambion mMessage mMachine transcription kit; 2 h; 37°C).

Site-Directed Mutagenesis. Serine residues 889, 890, 896, and 897 in the C1 cassette were mutated to alanine singly or altogether. To generate the mutant subunit $NR1_{100}(\Delta C2')$ (truncated just after C0), the codon for Asp-864 (first residue in C1) was replaced by a stop codon. To generate NR1(ΔC) ("$NR1_{stop838}$"; ref. 38), the codon for Arg-839 (six residues after the M4 domain) was replaced by a stop codon. To generate NR2A(ΔC), the codon for Thr-850 (13 residues after M4) was replaced by a stop codon. These truncations removed all serines and threonines downstream from M4 in the two subunits. Mutagenesis was performed with QuikChange (Stratagene), and codon substitutions were confirmed by DNA sequencing.

Electrophysiological Experiments in *Xenopus* Oocytes. Recombinant NR1/NR2A receptors were expressed in *Xenopus* oocytes as described (39). Selected Stage V and VI oocytes were injected with *in vitro* transcribed NR1 and NR2A mRNAs (1:2; 50 nl per cell). Samples of mRNA were diluted to achieve expression of receptors with maximal NMDA currents in the range of 100–300 nA. At 3–6 days after injection, responses were recorded in an extracellular solution of Mg^{2+}-free Ca^{2+} Ringer's solution as described (12). Because Ca^{2+} influx through the NMDAR can cause Ca^{2+} amplification (12) and inactivation (40), and activation of chloride channels endogenous to the oocyte (41) and thereby affect measurements of PKC potentiation, experiments were also performed in Ba^{2+} (nominally Ca^{2+}-free) Ringer's solution. Recording electrodes contained 3 M KCl; resistance was 0.5–2 MΩ. Currents were elicited by bath application of NMDA (300 μM with 10 μM glycine) at a holding potential of −60 mV. For maximal PKC activation, oocytes were incubated in TPA (100 nM; 10 min). Because of variability among batches of oocytes, only batches of oocytes showing an 8- to 12-fold potentiation of $NR1_{100}$/NR2A receptor responses were used.

Data Acquisition and Analysis. Current records were filtered at 1 kHz. In Ca^{2+} Ringer's solution, NMDA currents had an initial peak, followed by a minimum or plateau. Current amplitude was measured at the end of a 20-s agonist application. PKC potentiation is defined as the ratio of NMDA-elicited whole-cell current measured after TPA to that before TPA. Because amplitude of the basal (pre-TPA) NMDA current tended to increase with successive applications of agonist ("run-up"), $I_{control}$ was defined as the response to the first NMDA application. Run-up did not affect the amplitude of the TPA-potentiated response and was absent in Ba^{2+} Ringer's solution. Data are means ± SEM for a minimum of three experiments performed with different oocytes from at least two batches. Statistical significance was assessed by the unpaired Student's *t* test ($P < 0.05$; GRAPHPAD 2.0).

Results

Splicing in of the C1 Splice Cassette Reduces PKC Potentiation. The C1 cassette of the NR1 subunit is known to have target sites of PKC phosphorylation. Thus, we first examined PKC-induced potentiation of pairs of receptors that differed in the presence or absence of C1: $NR1_{100}$/NR2A vs. $NR1_{110}$/NR2A, $NR1_{101}$/NR2A vs. $NR1_{111}$/NR2A, $NR1_{000}$/NR2A vs. $NR1_{010}$/NR2A, and $NR1_{001}$/NR2A vs. $NR1_{011}$/NR2A (Fig. 1*A*, sample records; Fig. 1*B*, mean PKC potentiation; arrows connect receptor pairs and indicate effect of splicing in of C1).

The presence of the C1 cassette significantly reduced PKC potentiation of NMDA responses for all four pairs of receptors recorded in Ca^{2+} (Fig. 1 *A* and *B*, filled circles) or Ba^{2+} Ringer's solution (Fig. 1*B*, open circles). The reduction in potentiation caused by C1 was independent of the presence of the N1, C2, or C2′ cassettes (Fig. 1*B*). Moreover, the degree of potentiation of the NMDAR splice forms did not correlate with basal current amplitudes (Fig. 1*C*) or with efficiency of expression at the cell surface (15).

Phosphorylation of Ser-890 in C1 Reduces PKC Potentiation. To examine further the relation between PKC-induced phosphorylation and potentiation, we mutated four serine residues identified as targets of kinase phosphorylation (S889, S890, S896, S897; refs. 13 and 14), which are contained in the C1 cassette of the NR1 subunit. Altogether, five mutant receptors were examined: a mutant in which all four serines were mutated to alanines and four mutants in which one serine was mutated to alanine. Mutation of all four serines partially rescued PKC potentiation (Fig. 2 *B* and *C*). Of the possible sites of phosphorylation, only phosphorylation of Ser-890 disrupts receptor clustering at the membrane (14). The mutant receptor lacking Ser-890 ($NR1_{111}$ (S890A)/NR2A) showed potentiation to 7.9 ± 0.4 ($n = 6$) times control, a value comparable to the level for the four-serine mutant receptor (Fig. 2*C*). Mutation of any one of the other serines (Ser-889, Ser-896, or Ser-897) did not significantly alter the action of TPA, indicating that phosphorylation of these residues is not involved in PKC potentiation. Thus, about half of the effect of the C1 cassette on PKC potentiation is due to phosphorylation of Ser-890.

Alternative Splicing of Exon 22 Modestly Affects PKC Potentiation. To examine specifically the role of the C2 and C2′ cassettes on PKC potentiation of NMDA responses, we compared pairs of receptors that differed by the presence of C2′ vs. C2 (Figs. 1*A* and 3*A*). In C1-containing receptors, splicing in of C2 (replacing C2′ with C2) resulted in a small but not significant increase in PKC potentiation. In C1-lacking receptors, splicing in of C2 (replacing C2′ with C2) resulted in a modest decrease in PKC potentiation (significant only for $NR1_{100}$/NR2A vs. $NR1_{101}$/NR2A receptors in Ca^{2+}; $P < 0.05$).

To investigate PKC potentiation of a receptor lacking C1, C2, and C2′ cassettes, we engineered a truncated NR1 receptor, $NR1_{100}(\Delta C2')$ (Fig. 3*B*). The C-terminal domain of the $NR1_{100}(\Delta C2')$ subunit contained only C0, the initial 33 amino acids after M4. The $NR1_{100}(\Delta C2')$/NR2A mutant had dramatically reduced mean basal current amplitude relative to the corresponding C2′ or C2-containing receptors, $NR1_{100}$/NR2A and $NR1_{101}$/NR2A (not illustrated). This finding suggests that specific sequences within C2′ and C2 enhance basal current amplitude, possibly by attachment to a cytoskeletal protein expressed by the oocyte to promote receptor insertion. In Ba^{2+} (nominally Ca^{2+}-free) solution, the $NR1_{100}(\Delta C2')$/NR2A truncation mutant showed significantly increased potentiation relative to that of the $NR1_{100}$/NR2A (or $NR1_{101}$/NR2A) wild-type receptor ($P < 0.001$; no significance in Ca^{2+} solution; Fig. 3 *C* and *D*).

Truncation of the C-Terminal Domain of NR1 Does Not Alter PKC Potentiation. Because the C0 domain of the NR1 subunit is intracellular and may interact with proteins involved in anchoring and targeting of the receptor, a C-terminal deletion mutant [NR1(ΔC)] was constructed, and the effects of phorbol ester on

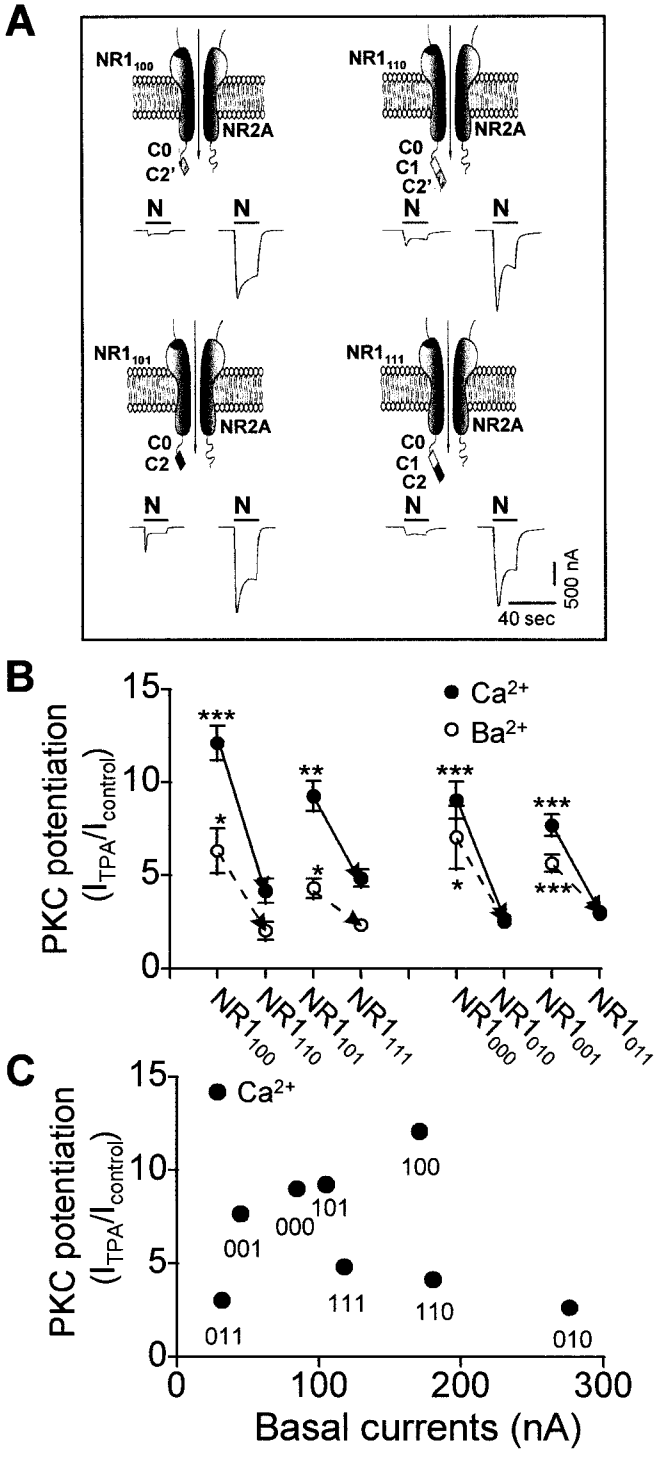

Fig. 1. Splicing in of the C1 cassette reduces PKC potentiation. NMDA responses recorded from *Xenopus* oocytes expressing NR1/NR2A receptors before and after treatment with TPA at a holding potential of −60 mV. (*A*) Currents recorded in Ca^{2+} Ringer's solution before (*Left*) and after (*Right*) treatment with TPA (100 nM; 10 min) from oocytes expressing four receptors: $NR1_{100}$/NR2A, $NR1_{110}$/NR2A, $NR1_{101}$/NR2A, and $NR1_{111}$/NR2A. The rows show receptors that differ by presence or absence of the C1 cassette; columns show receptors that differ by presence of C2′ vs. C2. (*B*) Mean PKC potentiation values for eight receptors arranged in pairs; arrows show the effect of splicing in of C1. C1-containing receptors showed reduced PKC potentiation (filled circles, Ca^{2+} Ringer's solution; open circles, Ba^{2+} Ringer's solution). For $NR1_{100}$/NR2A vs. $NR1_{110}$/NR2A, potentiation was to 12.1 ± 1.0 (n = 11) vs. 4.2 ± 0.7 (n = 6) times the control response in Ca^{2+} (P < 0.001; *Upper Right* vs. *Upper Left* records in *A*). For $NR1_{101}$/NR2A vs. $NR1_{111}$/NR2A, potentiation was to 9.3 ± 0.9 (n = 8) vs. 4.8 ± 0.5 (n = 5) times the control response (P < 0.01; *Lower Right* vs. *Lower Left* records in A). *, P < 0.05; **, P < 0.01; ***, P < 0.001. (*C*) The degree of PKC potentiation is not inversely related to basal current.

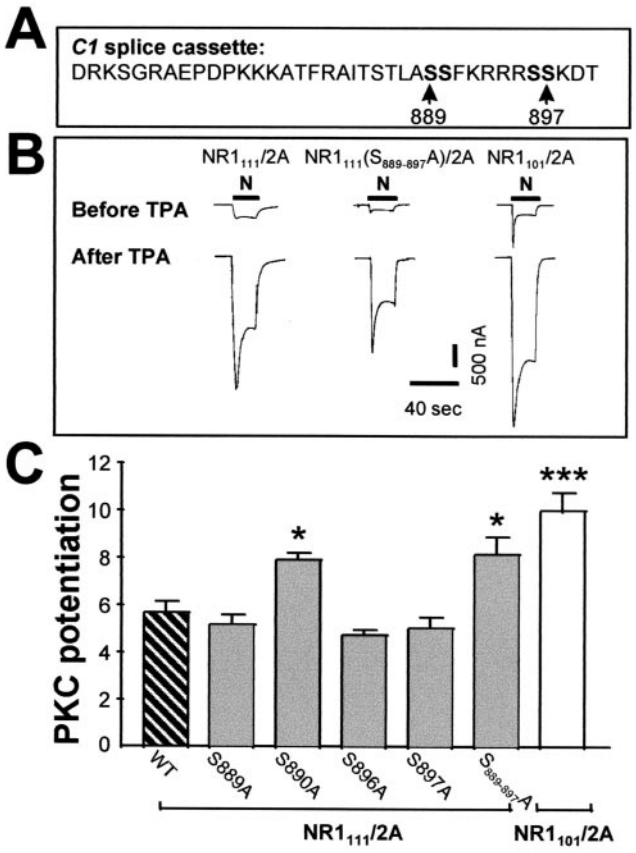

Fig. 2. Ser-890 in the C1 cassette reduces PKC potentiation. (*A*) Amino acid sequence of the C1 cassette of the NR1 subunit. Serine residues 889, 890, 896, and 897 were mutated to alanines, singly or in combination. (*B*) Currents recorded in Ca^{2+} Ringer's solution from oocytes expressing $NR1_{111}$/NR2A, $NR1_{111}(S_{889-897}A)$/NR2A, and $NR1_{101}$/NR2A receptors before (*Upper*) and after (*Lower*) incubation with TPA. Potentiation of $NR1_{111}(S_{889-897}A)$/NR2A receptors was greater than that for $NR1_{111}$/NR2A receptors. (*C*) Mean PKC potentiation of wild-type (WT) and mutant receptors in Ca^{2+} Ringer's solution. TPA potentiation of wild-type $NR1_{111}$, $NR1_{111}$(S889A), $NR1_{111}$(S890A), $NR1_{111}$(S896A), $NR1_{111}$(S897A), $NR1_{111}(S_{889-897}A)$/NR2A, and wild-type $NR1_{101}$/NR2A was to 5.7 ± 0.5 (n = 7), 5.2 ± 0.5 (n = 3), 7.9 ± 0.4 (n = 6; *, P < 0.05 vs. $NR1_{111}$/NR2A), 4.7 ± 0.3 (n = 3), 5.0 ± 0.5 (n = 3), 8.2 ± 0.8 (n = 7; *, P < 0.05 vs. $NR1_{111}$/NR2A), and 10.0 ± 0.8 [n = 6; ***, P < 0.001 vs. $NR1_{111}$; **, P < 0.01 vs. $NR1_{111}$(S890A) and $NR1_{111}(S_{889-897}A)$] times control, respectively.

NR1(ΔC)/NR2A receptors were examined (Fig. 4). This truncation mutant lacks the last five amino acid residues of the C0 region of the NR1 subunit, which have been shown to be required for Ca^{2+} inactivation (38).

The amplitude of the NMDA-elicited basal current of the NR1ΔC/NR2A mutant receptor was about two-fold greater than that of the wild-type receptor and did not have the initial peak current followed by desensitization that is a hallmark of the wild-type NMDAR in confirmation of results obtained by Krupp *et al.* (ref. 38; Fig. 4*A*, first and second rows). Moreover, the initial current measured in Ca^{2+} was greater than that measured in Ba^{2+} and increased during agonist application. In Ca^{2+} Ringer's solution, the mutant NR1ΔC/NR2A receptors showed

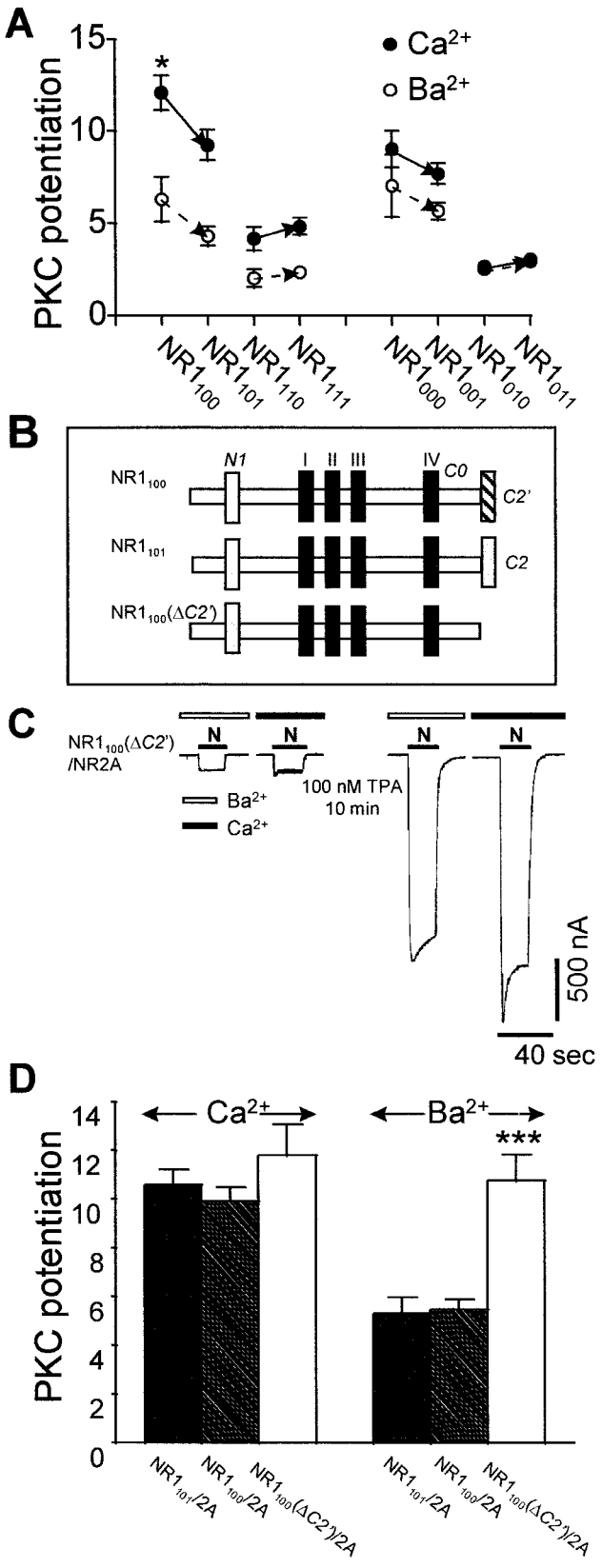

Fig. 3. Alternative splicing of exon 22 has little effect on PKC potentiation. (*A*) Fig. 1*C* replotted with receptor pairs connected by arrows to indicate effect of exchange of C2′ by C2. (*B*) Wild-type $NR1_{100}$ and $NR1_{101}$ and mutant $NR1_{100}(\Delta C2')$ subunits. (*C*) Currents recorded from oocytes expressing mutant $NR1_{100}(\Delta C2')$/NR2 receptors before and after TPA treatment. (*D*) Mean potentiation of $NR1_{101}$/NR2A, $NR1_{100}$/NR2A, and $NR1_{100}(\Delta C2')$/NR2A receptors. In Ca^{2+}, potentiation of $NR1_{101}$/NR2A, $NR1_{100}$/NR2A, and $NR1_{100}(\Delta C2')$/NR2A receptors was to 10.6 ± 0.7 (n = 9), 9.9 ± 0.6 (n = 24), and 11.8 ± 0.1.3 (n = 10) times control (differences not statistically significant). In Ba^{2+}, potentiation was to 5.3 ± 0.0.7 (n = 9), 5.5 ± 0.5 (n = 19), and 10.8 ± 1.1 (n = 10) times control (***, $P < 0.001$ vs. each wild-type receptor).

a significantly reduced degree of potentiation relative to that of the wild-type receptor (Fig. 4*B*). However, the amplitude of the potentiated current of the truncation mutant did not differ significantly from that of the wild-type receptor, and the increase in basal current observed during agonist application accounts for the apparent decrease in potentiation. These findings also suggest that in the presence of extracellular Ca^{2+}, a component of potentiation of the wild-type receptor is associated with relief of Ca^{2+} inactivation. In Ba^{2+} (nominally Ca^{2+}-free) solution, the C-terminal deletion mutant did not differ significantly from wild-type in either basal current or degree of potentiation (Fig. 4*B*).

Truncation of the C-Terminal Domain of NR2A Does Not Alter PKC Potentiation. The C terminus of the NR2A subunit serves as substrate for phosphorylation by PKC (17), CamKII (42, 43), and protein tyrosine kinases (44). A C-terminal deletion mutant [NR2A(ΔC)] was constructed by replacement of the codon for the 13th amino acid residue after M4 by a stop codon; this mutation removes all serines and threonines downstream from M4. The truncated receptor NR1/NR2A(ΔC) showed virtually the same degree of PKC potentiation as the wild-type receptor (Fig. 4 *A* and *B*). Because the mutant lacks the C terminus of NR2A, we considered whether PKC phosphorylates the $NR1_{100}$ (C1, C2-lacking) subunit. In studies involving immunoblotting of NR1 subunits expressed in HEK-293 cells, we failed to detect PKC-induced phosphorylation of sites other than those contained in C1 (n = 3; data not illustrated). Thus, PKC potentiation of NMDAR responses can occur without phosphorylation of the receptor itself.

Truncation of the C-Terminal Tails of NR1 and NR2A Reduces but Does Not Abolish PKC Potentiation. The double-truncation mutant NR1(ΔC)/NR2A(ΔC) had a reduced basal current and reduced degree of PKC potentiation relative to that of wild-type or of either single truncation mutant (Fig. 4*A*, fourth row). Thus, a significant component of potentiation can be mediated by the C terminus of either subunit, and the effects of the subunits are not additive.

Xenopus oocytes endogenously express a unitary glutamate receptor subunit termed XenU1 (45) and may express an NR2B homolog (12). To rule out the possibility that the oocyte provides a subunit or subunits that coassemble with rat NR1 to form functional NMDA channels, we carried out an mRNA "titration" experiment in which varying amounts of NR2A(ΔC) mRNA were coinjected with a constant amount of $NR1_{100}$ per oocyte. In oocytes injected with $NR1_{100}$ mRNA alone (i.e., 0 ng NR2A(ΔC)), NMDA elicited a small, desensitizing inward current, suggestive of coassembly of rat $NR1_{100}$ with XenU1 or other endogenous subunit to form functional channels. TPA potentiated responses of these receptors by 22-fold. As the ratio of NR2A(ΔC) to $NR1_{100}$ mRNA was increased, basal current increased by ≈10-fold, indicative of increased assembly and insertion of rat $NR1_{100}$/NR2(ΔC) receptors (and/or higher ratio of NR2 to NR1 per channel); the degree of PKC potentiation decreased to about 8-fold. The increase in basal current indicates that almost all the receptors were rat NR1/NR2A and that most of the potentiated current was from NR1/NR2A(ΔC)

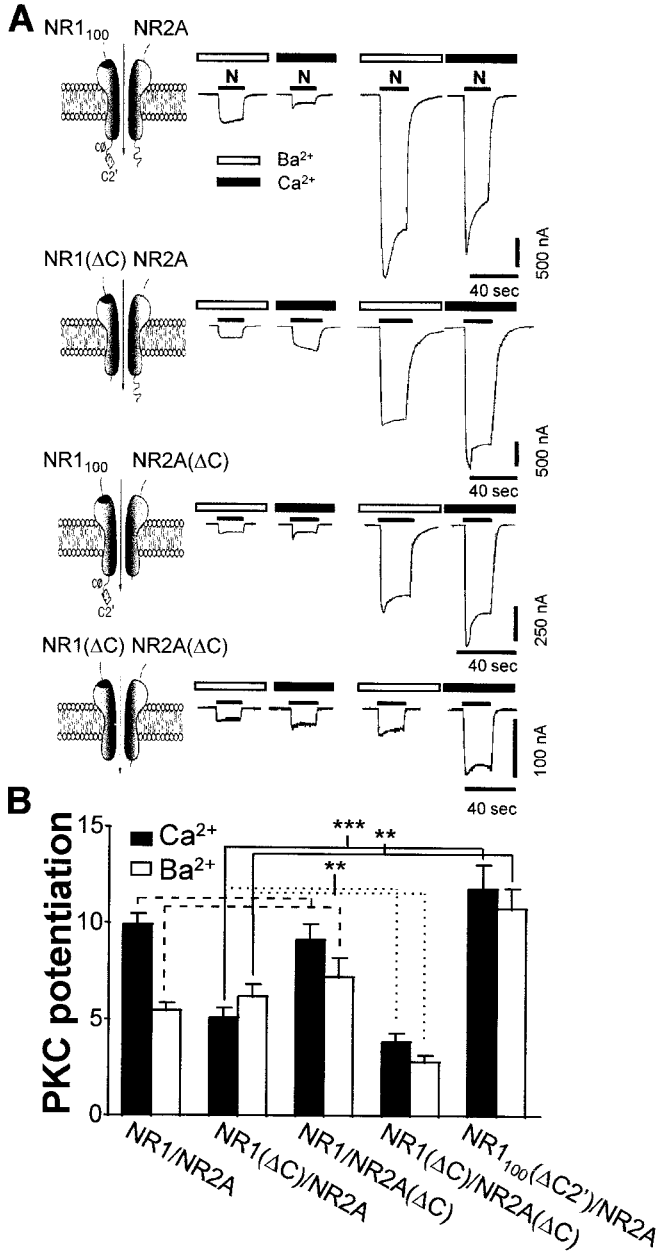

Fig. 4. The C-terminal tails of NR1 and NR2 are not required for PKC potentiation. (*A*) Responses of $NR1_{100}$/NR2A, NR1(ΔC)/NR2A, $NR1_{100}$/NR2A(ΔC), and NR1(ΔC)/NR2A(ΔC) receptors in Ba^{2+} and Ca^{2+} Ringer's solution before and after TPA treatment. Responses of NR1(ΔC)/NR2A receptors were larger in Ca^{2+} than in Ba^{2+} Ringer's solution and increased during agonist application. Basal currents of NR1/NR2A(ΔC)and NR1(ΔC)/NR2A(ΔC) receptors were small relative to those of wild-type receptors. (*B*) Mean PKC potentiation of mutant and wild-type receptors illustrated in *A* and of $NR1_{100}$(ΔC2′)/NR2A receptors. NR1(ΔC)/NR2A receptors showed significantly less potentiation than did wild-type $NR1_{100}$/NR2A receptors (which contain the C0 region and C2′ splice cassette) in Ca^{2+} Ringer's solution ($P < 0.001$) but did not differ significantly in Ba^{2+} Ringer's solution. The apparent reduction in the degree of potentiation could be accounted for by the larger basal current of the truncation mutant. NR1(ΔC)/NR2A receptors showed less potentiation than did $NR1_{100}$(ΔC2′)/NR2A receptors (which contain the C0 region) in Ca^{2+} ($P < 0.001$) and Ba^{2+} Ringer's solution ($P < 0.01$). Potentiation of $NR1_{100}$/NR2A(ΔC) receptors did not differ significantly from that of wild-type receptors. The double-truncation mutant NR1(ΔC)/NR2A(ΔC) showed significantly less PKC potentiation that did either single truncation mutant [$P < 0.01$ vs. NR1(ΔC)/NR2A and vs. $NR1_{100}$/NR2A(ΔC)]. PKC potentiated responses of $NR1_{100}$/NR2A, NR1(ΔC)/NR2A, $NR1_{100}$/NR2A(ΔC), NR1(ΔC)/NR2A(ΔC), and NR1(ΔC2′)/NR2A receptors to 9.9 ± 0.6 ($n = 24$), 5.1 ± 0.5 ($n = 16$), 9.1 ± 0.9 ($n = 7$), 3.9 ± 0.5 ($n = 6$), and 11.8 ± 0.1.3 ($n = 10$) times control in Ca^{2+} and to 5.5 ± 0.5 ($n = 19$), 6.2 ± 0.7 ($n = 11$), 7.2 ± 1.1 ($n = 7$), 2.8 ± 0.4 ($n = 6$), and 10.8 ± 1.1 ($n = 10$) times control in Ba^{2+} Ringer's solution. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

receptors rather than from heteromers assembled from rat NR1 and *Xenopus* endogenous subunits.

We also examined the effect of increasing the ratio of rat NR2A(ΔC):NR1(ΔC) mRNA. Injection of increasing amounts of NR2A(ΔC) with a constant amount of NR1(ΔC) mRNA (0:1 to 2:1) increased basal current by about 15-fold. In contrast, the degree of PKC potentiation decreased with increasing NR2A(ΔC) mRNA per oocyte over the range of 0:1 to 2:1 from 8-fold to 4-fold. Increasing the ratio of NR2A(ΔC) mRNA to NR1(ΔC) mRNA from 2:1 to 5:1 did not increase basal current further or reduce the degree of potentiation. Thus, the basal and potentiated currents could be attributed to activation of the double-truncation mutant and not of receptors containing a *Xenopus* subunit.

Discussion

The present study examined the role in PKC potentiation of residues, cassettes, and domains in the C-terminal tails of the NR1 and NR2A subunits. We report herein that splicing out of the C-terminal cassette C1, which contains the only known sites of PKC phosphorylation on the NR1 subunit (13, 14), increased PKC potentiation of NMDA responses. Mutation of Ser-890, which is implicated in targeting and clustering of NR1 subunits at the membrane (13, 14), also increased PKC potentiation but less so than splicing out of the entire cassette. Phosphorylation of Ser-890 could reduce potentiation by (*i*) direct inhibition of NMDAR channel activity or (*ii*) block of PKC-induced phosphorylation of sites on the NR2 subunits or associated proteins.

Splicing out of the C2 cassette, which is replaced by C2′, had little effect on either basal NMDA current or potentiation by PKC. Truncation of the C2′ (or C2) cassette in an NR1 subunit lacking C1 significantly reduced basal NMDA currents and modestly increased PKC potentiation (relative to that of wild-type $NR1_{100}$/NR2A or $NR1_{101}$/NR2A receptors) in Ba^{2+} Ringer's solution (but not in Ca^{2+} Ringer's solution). The positive contribution of the C2′ (or C2) cassette to basal current suggests the possible presence of a recognition sequence or sequences in this region required for binding to the receptor of membrane-associated insertion or anchoring proteins. Although the C2′ splice cassette contains a PDZ binding consensus sequence (46), studies involving the yeast two-hybrid system show no evidence for interaction between the NR1 C-terminal tail and either PSD-95 or SAP97 (47).

The C-terminal domains of the NR1 and NR2A subunits contain all known sites of PKC-, PKA-, and tyrosine kinase-induced phosphorylation (14, 17) and provide binding sites for a number of intracellular neuronal molecules implicated in receptor localization at the postsynaptic density (see above). Unexpectedly, truncation of the C-terminal domain of either the NR1 or NR2A subunit did not significantly alter PKC potentiation. Our finding that PKC potentiates $NR1_{100}$/NR2A(ΔC) receptors (which lack the C1 and C2 exon cassettes) demonstrates that potentiation of NMDAR responses occurs in the absence of any known phosphorylation sites.

PKC potentiation of NR1(ΔC)/NR2A receptors could occur by direct phosphorylation of the C terminus of NR2A (17) and/or of a member of the PSD-95/SAP90 family, which binds via the C-terminal tail of the NR2 subunit. Alternatively, potentiation could occur via activation of a member of the src family of tyrosine kinases, which phosphorylate serine residues

in the tail of NR2A. The observation that NMDARs in which both NR1 and NR2A subunits were truncated showed significant but reduced PKC potentiation shows that full potentiation requires the C-terminal tail of either NR1 or NR2A.

Our conclusion that PKC-induced potentiation of NMDARs does not occur via phosphorylation of the receptor subunits is unexpected. Presumably, PKC potentiation occurs via phosphorylation of an as yet unidentified accessory protein. It is possible that this protein leads to interaction with extracellular domains of the receptor subunits, by analogy to the extracellular immediate-early gene product Narp, which promotes insertion and/or stabilization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (48). Recent studies from our laboratory involving patch-clamp recording of NMDA single channel activity indicate that PKC-induced potentiation involves both an increase in channel open probability and insertion of new channel molecules at the cell surface (J. Y. Lan, A. Skeberdis, T. Jover, R. C. Araneda, Y. Lin, X.Z., R.S.Z., and M.V.L.B., unpublished observations). Findings from the present study indicate that neither mechanism requires direct phosphorylation of the NMDAR protein. PKC potentiation via phosphorylation of an accessory protein is likely to be an important mode of NMDAR regulation *in vivo* and may be involved in NMDA-dependent long-term potentiation (2).

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