Protein Kinase C Transiently Activates Heteromeric N-Methyl-d-aspartate Receptor Channels Independent of the Phosphorylatable C-terminal Splice Domain and of Consensus Phosphorylation Sites* 

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We have expressed dual subunit combinations of isoforms of the N-methyl-d-aspartate receptor, NR1A-NR2A and NR1C-NR2A, in Xenopus oocytes. We show that both forms of the receptor are stereospecifically activated by low concentrations (10 nM) of the phorbol ester 4β-phorbol 12-myristate 13-acetate, known to activate protein kinase C (PKC). The activation is transient, and, after reaching a maximum in about 10 min, it decreases rapidly in spite of the continuous presence of phorbol ester. The addition of 2 μM oleoylacylglycerol had similar consequences. NR1C differs from NR1A by a deletion of 37 amino acids that include four consensus phosphorylation sites for PKC in the C-terminal region. The corresponding peptide has been shown to become phosphorylated upon activation of PKC in neurons (Tingley, W. G., Roche, K. W., Thompson, A. K., and Huganir, R. L. (1993) Nature 364, 70-73). However, the activity of NR1C-NR2A receptors was stimulated 7-fold, twice the potential observed for NR1A-NR2A. By site-specific mutagenesis of NR1C and NR2A, we removed additional consensus PKC phosphorylation sites located between TM3 and TM4. Coexpression of these mutant subunits showed a similar response to phorbol esters as wild type receptors. Our results indicate that neither the predicted consensus phosphorylation sites between transmembrane sequences TM3 and TM4 nor the phosphorylatable C-terminal splice domain is essential for the modulation of N-methyl-d-aspartate receptors by PKC.

Glutamate is the major excitatory neurotransmitter in vertebrate brain where it acts as an agonist at ligand-gated ion channels and at metabotropic receptors. Glutamate neurotransmission has been implicated in neuronal plasticity and in neurodegeneration (for review, see Ref. 1). NMDA receptors constitute a subgroup of ligand-gated glutamate channels, which are essential for inducing long-term potentiation and, due to their Ca2+ permeability, for the neurotoxicity of glutamate. cDNAs encoding a large number of protein subunits have recently been cloned (2-9). NR1 subunits exhibit on their own small ion currents after functional expression in heterologous systems (2), while NR2 subunits do not result in such an expression (4, 5). Combined expression of NR1 subunits with NR2 subunits, called δ and ε in mouse, show large ion currents (4, 5), suggesting a heterooligomer structure for NMDA receptor channels.

Evidence has been provided that PKC enhances NMDA-mediated current responses (3, 6, 8, 10-12). Intracellularly applied PKC enhances such currents, in part by reducing the Mg2+-dependent block in isolated trigeminal neurons (10), and phorbol esters stereoselectively enhance NMDA-induced currents measured in Xenopus oocytes after injection with rat brain RNA (11). This potentiation of NMDA-induced currents is probably an important step in the induction of long-term potentiation (13), a phenomenon thought to underlie memory and learning.

On the molecular level, it is not clear how the interaction of PKC with NMDA receptors occurs. Specifically, it is not known which PKC isoform is involved and what amino acids on NMDA receptors are responsible for the functional modulation. It has been shown that homomeric NMDA receptor function can be enhanced by β-PMA after expression in Xenopus oocytes. This is the case for mouse 1δ (homologous to 1A subunits from rat) (6), rat 1A, and rat 1G subunits (called NR1B in the nomenclature chosen here) (8). Similarly, the function of 1ε1 and 1ε2 but not 1ε3 heteromeric NMDA channels has been reported to be enhanced by phorbol ester (6). In some of these experiments, it has been noted that the stimulation by β-PMA was transient (6, 12), but, because β-PMA was always added only at the beginning of the experiments, the loss of stimulation might have been caused by the washout of β-PMA.

Very recently, Tingley et al. (14) suggested that a specific C-terminal splice domain occurring in some variants of the NR1 subunits, carrying several consensus phosphorylation sites for PKC, is responsible for the functional modulation of NMDA receptors by PKC. In addition, they proposed that alternative splicing of subunits might add to diversity in long-and short-term modulation of synaptic efficiency. Structural evidence was provided for the use of the phosphorylation sites on the corresponding amino acids.

We describe here in detail the modulation of heteromeric NMDA receptor channels after continuous stimulation of PKC by β-PMA and OAG and compare the effects of the presence or the absence of the C-terminal splice domain. Furthermore, in the heteromeric channel composed with the NR1C subunit, which is lacking the 37 amino acids excised by exon 21 (15), we removed all four putative substrate sites for PKC located on the putative intracellular domains of the two subunits between TM3 and TM4 and studied the functional consequences.

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§ The abbreviations used are: NMDA, N-methyl-d-aspartate; PKC, protein kinase C; OAG, 1,2-oleoylacylglycerol; β-PMA, 4β-phorbol 12-myristate 13-acetate; ε-PMA, 4ε-phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction.

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MATERIALS AND METHODS

Isolation of cDNAs—To isolate the NR1A and NR1C cDNAs, whole rat brain poly(A) RNA (1 μg) was reverse-transcribed to cDNA and then amplified by means of PCR (using a gene Amp RNA-PCR kit from Perkin-Elmer). For PCR, the following oligonucleotide primers derived from the rat NMDAR1A sequence (2) were used: sense, 5'-TTCTCTA-CTCACTCTGCCCCAGGCTCAG-3'; antisense, 5'-GAACCTGCGCCATCTTCTCGAAAA-3'. The final PCR reaction product was extracted with phenol/chloroform and then precipitated with ethanol. The PCR products were electrophoresed on a 1% agarose gel. The major band of 513 base pairs was cut from the gel. The PCR-derived fragment was 32P-nick-translated and used as a probe for hybridization screening of a rat brain cDNA library. Among 1.2 × 10⁶ recombinants examined, 14 positive clones were plaque-purified. The sequence analysis of the two largest inserts (designated NR1A and NR1C) revealed that both clones are identical to the rat NMDAR1A and 1C subunits described by Sugihara et al. (7).

To isolate the NR2A subunit, a rat cortex A458 cDNA library was screened with a 675-base pair rat cDNA probe. This probe was obtained from reverse transcription PCR amplification of rat brain poly(A) RNA using PCR primers based on the sequence of NR2A published by Monyer et al. (5) (sense, 5'-TACTCTACATGCTCAATGAGGAACG-3'; antisense, 5'-GATACGAGGTGACGATCGAG-3'). The NR2A cDNA clone was then constructed by ligating two overlapping clones at the unique KpnI site.

Site-directed Mutagenesis of PKC Consensus Phosphorylation Sites—The cDNAs encoding the NR1C and NR2A subunits were subcloned into the M13 mp 18 vector (Pharmacia LKB Biotechnology Inc.). Site-directed mutagenesis was performed (16) using the single-stranded DNA as a template and the following oligonucleotide primers (synthesized by Genosys Biotechnologies Inc., Texas): NR1C (S658A), 5'-AGATGAACTTGTCTGCGGGGTITCTGAGCC-3'; NR1C (S747A), 5'-TCTGCTTCCAGGGGGzGTCC?TGCGCATGCC-3'; NR2A (S773A), 5'-AGATGAACTTGTCTGCGGGGTITCTGAGCC-3'; NR2A (S976A), 5'-TCTGCTTCCAGGGGGzGTCC?TGCGCATGCC-3'. The number of amino acids corresponds to the mature proteins. Double and triple mutations of PKC sites were successively introduced into the NR1C cDNA. The complete DNA inerts of all the mutants were sequenced to ensure that the correct mutants were introduced without unwanted side mutations.

Expression of NMDA Receptors in Xenopus Oocytes—The in vitro transcription, capping, and polyadenylation of the rat brain subunit isoforms have been described elsewhere (17-19). The cRNA combinations were coprecipitated in ethanol, shipped, and stored below 0 °C. Isolation of follicles from the frogs, culturing of the follicles, injection with cRNA, and removal of the follicular cell layers from the oocytes were all performed as described earlier (20). Follicles were injected with about 50 nl of the capped transcripts. The solution contained the transcripts coding for each of the different subunits at a concentration of 20 nm to allow injection of stoichiometric amounts. The follicular cell layers were removed from the oocytes 1 day or 2 days prior to the electrophysiological experiments.

Electrophysiological Experiments—All electrophysiological measurements were carried out on denuded oocytes. Oocytes were placed in a 0.4-mM bath on a nylon grid, and the bath was perfused throughout the experiment at 6 ml/min with 90 mM NaCl, 1 mM KCl, 1 mM CaCl₂, 5 mM Hepes-NaOH (pH 7.4). The perfusion medium could be switched (70%) to allow injection of stoichiometric amounts. The follicular cell layers were removed from the oocytes 1 day or 2 days prior to the electrophysiological experiments.

Fig. 1. Effect of β-PMA on NMDA-induced currents mediated by NR1C-NR2A expressed in Xenopus oocytes. After several control responses to ensure constant current response, 10 nm β-PMA was applied for 1 min at time 0 and again for 1 min out of 4 during 30 min. This procedure was chosen in order to prevent both accumulation and washout of the hydrophobic drug. 100 μM NMDA/10 μM glycine (bar) was applied for 30 s every 4 min in order to measure the current amplitude. The times given above the bars indicate the time measured from the first exposure to β-PMA.

Nomenclature of NMDA Receptor Subunits—In this paper, we are following the nomenclature suggested by Sugihara et al. (7) and Monyer et al. (5) and use the names NR1 and NR2 for the subunit groups also called NMDAR1 and NMDAR2 for rat subunits and ζ and ε for mouse subunits. The present work was performed with the subunit isoforms NR1A, NR1C, and NR2A. NR1C differs from NR1A by the deletion of 37 amino acid residues (D884-T920) encoded by exon 21 (7, 15) containing four consensus phosphorylation sites for PKC.

RESULTS

Functional NMDA Receptor Expression in Xenopus Oocytes—The dual subunit combination NR1C-NR2A was expressed in Xenopus oocytes. Maximal current amplitudes amounted to 100–400 nA in a Ca-free medium. In the medium containing the agonists 100 μM NMDA and 10 μM glycine, Ca²⁺ ions were replaced by Ba²⁺ ions in order to eliminate Ca²⁺-activated currents from the oocyte. Ca²⁺ was maintained in the medium during the intervals between agonist application in order to prevent depletion of Ca²⁺ in the oocyte. Agonist-induced desensitization was rapidly reversed within less than 1 min, such that our observations are not perturbed by desensitization.

Response of NR1C-NR2A Type NMDA Receptors to PMA and OAG—Oocytes expressing NR1C-NR2A were exposed to conditions known to result in activation of PKC. β-PMA was repeated applied for 1 min with intervals of 3 min in order to minimize both accumulation and washout during the experiment. Fig. 1 shows a typical experiment. After several control applications of NMDA/glycine to ensure constant current amplitudes, 10 nm β-PMA was applied for 1 min. Within 2 min after the first exposure to β-PMA, the NMDA current amplitude was more than doubled, and, with about 10 min, a maximum amplitude was reached. At later times, current amplitudes decreased again. Fig. 2 shows the time course of the
Current amplitudes were determined as described in the legend to Fig. 1, except for the experiment including staurosporine in which the drug application lasted only 10 min. Data are shown as mean ± S.D. (n = 4, except for PMA/staurosporine, where n = 3).

β-PMA effect averaged from four oocytes of the same batch. The rising phase of the current response was studied in four additional batches of oocytes originating from three additional donor animals. Similar averaged data were obtained in all cases, using two to four oocytes from each batch. Some variation of the observed current decrease from the maximal current response was found between different batches of oocytes. The average current amplitudes in a total of five batches, 30 min after the first exposure to β-PMA, were 110% (3 oocytes), 547% (5 oocytes), 156% (4 oocytes) (Fig. 2), 115% (3 oocytes), and 162% (2 oocytes) of the response before application of β-PMA. In five oocytes from four different batches of oocytes, the response after 30 min was smaller than the initial response before application of β-PMA. The reason for the observed current decrease, in spite of the repeated application of β-PMA, is not clear from our experiments.

In control experiments (not shown), the amplitude of NMDA-induced currents remained constant for more than 30 min, indicating that the current decrease mentioned above is not due to a run-down of NMDA receptor channels. The stereoisomeric control substance α-PMA (100 μM) that does not activate PKC at a 10-fold higher concentration than β-PMA did not affect the NMDA current amplitude (Fig. 2). If the nonspecific protein kinase inhibitor staurosporine (5 μM) was applied in conjunction with β-PMA, the stimulation was strongly reduced from about 700 to 150% (Fig. 2) of the control current amplitude (100%). Surprisingly, staurosporine alone also slightly stimulated the control response. Staurosporine at 1 μM was also able to largely suppress β-PMA effects (not shown). Continuously applied OAG (2 μM), a synthetic diacylglycerol, had very similar effects as β-PMA on NMDA-induced currents, except that the onset of action was somewhat slower (Fig. 3). The effects of α-PMA and OAG were confirmed in additional experiments.

**Effect of the C-terminal Splice Domain on the Modulation by PKC**—In similar experiments, the effect of β-PMA on the NR1A-NR2A receptor channel was determined. The additional C-terminal amino acids did not significantly affect the response to the agonists (not shown). Fig. 4A shows the time course of the β-PMA effect in this subunit combination. Stimulation of the control current amplitude was about half that observed with NR1C-NR2A in the same batch of oocytes (Fig. 4A). In three such experiments, relative average stimulation of NR1A-NR2A compared with NR1C-NR2A was 54, 51, and 62%. Subunit NR1C differs from NR1A by the absence of 37 amino acids encoded by exon 21 (15) containing four consensus phosphorylation sites for PKC. Our data show, seemingly paradoxically, that removal of these sites increases the extent of activation by PKC.

**Basal NMDA Receptor Activity**—We find that PKC activates NMDA receptor channels. It may be thought that full activity of

![Figure 2](image1.png)

**Fig. 2.** Time dependence. Effects of 10 nM β-PMA (●), 10 nM β-PMA/5 μM staurosporine (■), and 100 nM α-PMA (○) on NMDA-induced currents mediated by NR1C-NR2A expressed in Xenopus oocytes. Current amplitudes were determined as described in the legend to Fig. 1, except for the experiment including staurosporine in which the drug application lasted only 10 min. Data are shown as mean ± S.D. (n = 4, except for PMA/staurosporine, where n = 3).

![Figure 3](image2.png)

**Fig. 3.** Time dependence of the effect of 2 μM OAG. The effect of a continuous exposure of OAG on NMDA-induced currents mediated by NR1C-NR2A expressed in Xenopus oocytes was determined as described in the legend to Fig. 1. Data are shown as mean ± S.D. (n = 3).
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DISCUSSION

We have studied in detail the modulation of heteromeric NMDA channels with the composition NR1C-NR2A by agents that activate PKC. So far, very little is known about the modulation of heteromeric NMDA channels. A study (6) with mouse \( \xi 1_1, \xi 1_2, \) and \( \xi 1_3 \) channels reported modulation of these channels by \( \beta \)-PMA in the presence of \( \xi 1 \) and \( \xi 2 \) but not in the presence of \( \xi 3 \). The \( \xi \) family of subunits in mouse corresponds to the NR2 family in rat. We show that the NR1C-NR2A current is stimulated after application of OAG (Fig. 3) and stereospecifically after the application of very low concentrations of \( \beta \)-PMA (Figs. 1 and 2). Stimulation by \( \beta \)-PMA occurs very rapidly (<2 min) but is strongly inhibited by the simultaneous application of the nonspecific kinase inhibitor staurosporine (Fig. 2). Together with the finding that \( \beta \)-PMA increases phosphorylation of NMDA receptors (14), our observations suggest that the modulation may be mediated by direct effects of PKC. But, on the basis of our experiments, we cannot exclude an indirect action of PKC on NMDA receptors via other proteins.

Transient Stimulation—We observe that the stimulation by activators of PKC is transient, reaching a maximum of about 6–7-fold at about 10 min (Fig. 2). Subsequent decline of the response was somewhat variable and reached, in some cases, amplitudes below the initial control response within 30 min. In our protocols, we ensured a continuous presence of activators of PKC. A transient stimulation of the NMDA-induced currents has recently been reported (6, 12), but, in these cases, high concentrations of \( \beta \)-PMA were applied initially only, followed by a washout period. Thus, it is not clear whether the transient nature of the stimulation was caused by the washout of \( \beta \)-PMA.

Our work can only suggest some hypotheses for the mechanism leading to the transient nature of the stimulation of NMDA-induced currents by PKC. It is possible that the isofonn of PKC involved is rapidly down-regulated and the NMDA channel dephosphorylated by a protein phosphatase within the duration of our experiment. Alternatively, the overphosphorylation of NMDA receptors could inhibit the channel function. Finally, PKC could turn on another enzyme acting in an inhibitory fashion on NMDA channels.

Role of the C-terminal Phosphorylation Sites—A previous biochemical experiment has shown that the NR1A subunit transfected into a permanent cell line is phosphorylated in the basal state and that this phosphorylation can be enhanced by exposure of the cells to \( \beta \)-PMA (14). \( \beta \)-PMA-induced phosphorylation was strongly reduced after deletion of C-terminal amino acids (construct identical to NR1C) or after converting four serine residues of PKC consensus phosphorylation sites to alanine (14). Such findings strongly suggested the involvement of these amino acids in the modulation of NMDA receptors by PKC. The fact shown here that NMDA receptors containing the NR1C subunit can be functionally modulated by PKC is in itself interesting. Previously, it has been shown that homomeric channels made of subunits lacking the C-terminal amino acid domain can also be stimulated by \( \beta \)-PMA (12). These observations are unexpected in the light of the biochemical work (14) and show that the four mentioned serine residues are dispensable for the modulation of NMDA channels by PKC. Direct comparison of NR1A-NR2A and NR1C-NR2A channels showed that removal of the extra amino acids does not lead to loss of stimulation by PKC but, paradoxically, to an increased stimulation.

The Phosphorylation Sites Lacking in NR1C May Be Involved in Defining Basal Activity—As indicated in the previous section, one could assume that full activity of the NMDA channels is revealed only after stimulation of PKC and that the C-terminal splice domain is involved in defining the basal ac-

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tivity. It is therefore interesting to compare this result with that of a recent publication on homomeric NMDA receptors in Xenopus oocytes and their modulation by PKC (12). The basal activity can be calculated from the data on the maximal stimulation by β-PMA. In spite of the different experimental protocols, the basal activity of the NR1A channel (33%) compares quantitatively very well with the basal activity value found here for NR1A-NR2A channels (30%). If our model is correct, it can be deduced from the data of Durand et al. (12) that about two-thirds of the basal activity is contributed by the presence of the first and about one-third by the second C-terminal splice domain.

We do not show here whether the enhanced basal activity of the receptor containing the additional 37 amino acids is a consequence of a phosphorylation of the additional sites. If it is the case, the basal phosphorylation does not necessarily have to occur by an isoform of PKC but could be achieved via another protein kinase recognizing the additional amino acids in NR1A. It should be borne in mind that our data were collected in Xenopus oocytes and that the steady state of basal phosphorylation and activity could be different in neurons.

Role of the Consensus Phosphorylation Sites for PKC on the Intracellular Loops between TM3 and TM4—We chose the subunit combination NR1C-NR2A for further work. In analogy to other ligand-activated ion channels, we assumed an involvement of consensus phosphorylation sites located on the putatively intracellular regions between TM3 and TM4 of the two subunits. Three such possible phosphate-accepting amino acids on NR1C and one on NR2A were replaced by alanine. In our assays, the mutant subunit combination showed a behavior very similar to that of the wild type combination, indicating that the four amino acids are not involved in the modulation of NMDA receptors by PKC.

In conclusion, we present here a detailed study of the modulation of heteromeric NMDA receptor channels by PKC. We show that the transient nature of the stimulation is a true effect, which is not just due to the washout of stimulators of PKC. We provide evidence that the modulation can also occur in the absence of the amino acids encoded in exon 21 (7, 15), which have been shown to be phosphorylated by PKC in neurons (14). However, we put forward the notion that the extra amino acids in NR1A may help to determine the basal activity of the channel. Four predicted phosphorylation sites between TM3 and TM4 are not required for the modulation or determination of basal activity. Thus, the molecular basis of the modulation of NMDA channel by PKC remains to be established.

Note Added in Proof—After final submission of this manuscript, a paper by Yamakura, T., Mori, H., Shimoji, K., and Mishina, M. (Biochem. Biophys. Res. Commun. 196, 1537-1544 (1993)) came to our attention. They report similar findings concerning the lack of a role of the C-terminal domain of the mouse (1 subunit.

Acknowledgments—We are grateful to Prof. H. Reuter, in whose laboratory functional expression studies were carried out, for continuous support and, together with Drs. V. Niggli and S. Kellenberger, for helpful discussions. We thank V. Gyoefly and V. Kiefer for skillful technical assistance.

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