Ca²⁺ Influx Amplifies Protein Kinase C Potentiation of Recombinant NMDA Receptors

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Protein kinase C (PKC) potentiates NMDA receptors in hippocampal, trigeminal, and spinal neurons. Although PKC phosphorylates the NMDA receptor subunit NR1 at four residues within the C terminal splice cassette C1, the molecular mechanisms underlying PKC potentiation of NMDA responses are not yet known. The present study examined the role of Ca²⁺ in PKC potentiation of recombinant NMDA receptors expressed in *Xenopus* oocytes. We found that Ca²⁺ influx through PKC-potentiated NMDA receptors can further increase the NMDA response ("Ca²⁺ amplification"). Ca²⁺ amplification required a rise in intracellular Ca²⁺ concentration at or near the intracellular end of the channel and was independent of Ca²⁺-activated Cl[−] current. Ca²⁺ amplification depended on extracellular Ca²⁺ concentration during NMDA application and not during PKC activation. Ca²⁺ amplification was reduced by the membrane-permeant Ca²⁺-chelating agent BAPTA-AM. Mu-

tant receptors with greatly reduced Ca²⁺ permeability did not exhibit Ca²⁺ amplification. Receptors containing the NR1 N-terminal splice cassette showed more Ca²⁺ amplification, possibly because of their larger basal current and therefore greater Ca²⁺ influx. Contrary to expectation, splicing out the two C-terminal splice cassettes of NR1 enhanced PKC potentiation in a manner independent of extracellular Ca²⁺. This observation indicates that PKC potentiation does not require phosphorylation of the C1 cassette of the NR1 subunit. PKC potentiation of NMDA receptors *in vivo* is likely to be affected by Ca²⁺ amplification of the potentiated signal; the degree of amplification will depend in part on alternative splicing of the NR1 subunit, which is regulated developmentally and in a cell-specific manner.

Key words: protein kinase C; NMDA receptors; alternative RNA splicing; TPA; BAPTA; *Xenopus* oocyte

The NMDA class of glutamate receptors plays a critical role in synaptic plasticity, formation of neuronal circuitry, and excitotoxicity (for review, see Choi and Rothman, 1990; Constantine-Paton, 1990; Bliss and Collingridge, 1993). Protein phosphorylation and dephosphorylation are thought to be important mechanisms of regulation of synaptic activity. The PKC-activating phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) enhances whole-cell currents (Gerber et al., 1989; Aniksztejn et al., 1992; Chen and Huang, 1992) and channel open probability (Chen and Huang, 1992) of NMDA receptors in hippocampal, trigeminal, and spinal neurons. TPA also potentiates NMDA responses in *Xenopus* oocytes expressing rat brain mRNA (Durand et al., 1992; Kelso et al., 1992; Urushihara et al., 1992) and recombinant NMDA receptors (Durand et al., 1992, 1993; Kutsuwada et al., 1992; Yamazaki et al., 1992; Mori et al., 1993). Agonists of μ opioid receptors (Chen and Huang, 1991), phosphoinositol-coupled metabotropic glutamate receptors (Aniksztejn et al., 1991, 1992; Kelso et al., 1992; Shen et al., 1995), and muscarinic acetylcholine receptors (Markram and Segal, 1990) potentiate neuronal and recombinant NMDA receptors via activation of PKC. PKC phosphorylates specific residues in the C1 splice cassette in the cytoplasmic C terminus of the NMDA

receptor subunit NR1 (Tingley et al., 1993). Phosphorylation at these sites may regulate receptor interactions with the cytoskeleton and clustering at the membrane (Ehlers et al., 1995) but may decrease PKC potentiation (Durand et al., 1993; Sigel et al., 1994).

Ca²⁺ modulates NMDA receptor activity by several mechanisms. Ca²⁺ reduces single-channel conductance by binding in the ion channel (Jahr and Stevens, 1993). Ca²⁺ influx reduces open probability without change in single-channel conductance (producing "slow inactivation") (Legendre et al., 1993; Rosenmund et al., 1995). Binding of Ca²⁺-calmodulin to the cytoplasmic C terminus decreases open probability and may contribute to Ca²⁺-dependent inactivation (Ehlers et al., 1996). High extracellular Ca²⁺ was reported to increase responses of recombinant NMDA receptors expressed in *Xenopus* oocytes (Koltchine et al., 1996).

NMDA receptors are assembled from NR1 and NR2 subunits (for review, see Nakanishi, 1992; Westbrook, 1994; Mori and Mishina, 1995). The NR1 subunit is encoded by a single gene, and alternative RNA splicing gives rise to eight possible splice variants (Sugihara et al., 1992). NR2 subunits are of four types, NR2A–D, encoded by four different genes (Meguro et al., 1992; Monyer et al., 1992). NMDA receptors are likely to be NR1/NR2 heteromeric complexes in the nervous system (Sheng et al., 1994; Behe et al., 1995; Blahos and Wenthold, 1996). Although injection of NR1 subunit mRNA into oocytes leads to formation of functional receptors with different properties dependent on the splice variant (Durand et al., 1993; Zhang et al., 1994; Zukin and Bennett, 1995), oocytes may provide an NR2 subunit. Oocytes provide a necessary subunit for two other heteromeric channels (Buller and White, 1990; Hedin et al., 1996).

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In the present study, we analyzed the effect of extracellular Ca^{2+} on PKC potentiation of recombinant NMDA receptors expressed in *Xenopus* oocytes. PKC potentiation of specific splice variants was greater when measured in 1 mM Ca^{2+} than when measured in 1 mM Ba^{2+} . The increase in potentiation observed in extracellular Ca^{2+} required that the Ca^{2+} be present during opening of the NMDA receptor, not during activation of PKC. The findings are consistent with a process of Ca^{2+} -dependent amplification in which Ca^{2+} enters through the potentiated NMDA receptor and binds to its inner face or a closely associated molecule to increase channel open time or conductance. Modulation of PKC potentiation of neuronal NMDA receptors by extracellular Ca^{2+} and by alternative splicing could have important roles in synaptic plasticity and in formation of neural circuitry.

MATERIALS AND METHODS

Site-directed mutagenesis. Site-directed mutations were made with the oligonucleotide-directed *in vitro* mutagenesis system, version 2 (Amersham, Arlington Heights, IL) as described (Zheng et al., 1994). In brief, NR1₀₁₁ and NR1₁₀₀ (nomenclature of NR1 splice variants according to Durand et al., 1993) were subcloned into the pBluescript SK(–) vector and used to transform the *Escherichia coli* strain DH5 α /IQ (Life Technologies, Gaithersburg, MD). Single-stranded DNA template was rescued with M13K07 helper phage (Bio-Rad, Hercules, CA). To make the channel mutants NR1₀₁₁(N598R) and NR1₁₀₀(N619R), we engineered a single codon substitution into the cloned NR1₀₁₁ and NR1₁₀₀ cDNAs to generate subunits carrying an arginine in place of asparagine in the second membrane domain M2. The following oligonucleotide was used for introduction of this change into the coding sequence 5'-CCTTCCCCAATGCCGAGCGGAGCAGGACGCC-3'. To engineer the channel mutant NR1₁₁₁(N619R), a *BsmI*–*Bam*HI fragment containing exons 21 and 22 was shuttled from wild-type NR1₁₁₁ into the mutant NR1₁₀₀(N619R) cDNA.

To introduce *N1* insert mutations with reduced positive charges in the NR1₁₁₁ receptor, a *BsmI*–*Bam*HI fragment containing exons 21 and 22 was shuttled from wild-type NR1₁₁₁ into the mutant M1, M2, and M3 (NR1₁₀₀) cDNAs (Zheng et al., 1994). Mutant NR1₁₁₁ receptors were as follows: N1–1 (K192A, K193A, and R194A), N1–2 (R207A, R208A, and K211A), and N1–3 (K192A, K193A, R194A, R207A, R208A, and K211A). Codon substitutions were confirmed by DNA sequencing across mutationally altered and ligation regions with Sequenase version 2.1 (United States Biochemical, Cleveland, OH).

RNA synthesis. NR1₀₁₁ cDNA was a gift from Dr. S. Nakanishi (Kyoto University, Kyoto, Japan); NR1₁₁₁ cDNA was a gift from Dr. V. R. Anantharam (University of Massachusetts, Worcester, MA) and subcloned to pBluescript SK(–) vector; NR1₁₀₀ cDNA was cloned in this laboratory (Durand et al., 1992). Mutant NR1 receptors were generated as described above. NR2A was a gift from Dr. M. Mishina (Niigata University, Niigata, Japan). To generate templates for transcription, circular plasmid cDNAs were linearized with *NotI* (wild-type and mutant NR1₀₁₁, NR2A) or *Bam*HI (wild-type and mutant NR1₁₀₀, NR1₁₁₁). Transcription reactions were performed with T7 and T3 polymerase (Ambion MEGAscript transcription kit, 4 hr at 37°C) in the presence of capped analog or a mMessage mMachine transcription kit (2 hr at 37°C).

Electrophysiological experiments in *Xenopus* oocytes. Adult female *Xenopus laevis* (Xenopus I, Ann Arbor, MI) were anesthetized by immersion in ice water or 0.15% aminobenzoic acid ethyl ester, and oocytes were isolated and prepared as described (Kushner et al., 1988, 1989). Selected stage V and VI oocytes were injected with *in vitro* transcribed RNA (~20 ng/cell; for heteromeric receptor expression, NR1 and NR2 were mixed in a ratio of 1:3) and maintained at 18°C in culture buffer (in mM: 103 NaCl, 2.5 KCl, 2 MgCl₂, 2 CaCl₂, and 5 HEPES, pH 7.5). Three to 7 d after injection, oocytes were clamped at –60 mV in Mg²⁺-free Ca²⁺ Ringer's solution (in mM: 116 NaCl, 2.0 KCl, 1.0 CaCl₂, and 10 HEPES, pH 7.2), unless otherwise specified, with a two-electrode voltage-clamp amplifier (Dagan or Axon Instruments). NMDA (300 μM) together with glycine (10 μM) was bath-applied to elicit responses. Current amplitude was measured at the minimum immediately after the initial peak (indicated by arrows in sample records in Figs. 1, and 5). In some experiments, 1.0 mM CaCl₂ was replaced by 1.0 mM BaCl₂ (Ba^{2+} Ringer's solution), or the concentration of CaCl₂ was changed. In experiments involving 5 and 10 mM Ca²⁺ Ringer's solution,

10 and 20 mM Na⁺-gluconate was substituted for equimolar NaCl to maintain constant concentrations of Na⁺ and Cl[–].

TPA (1 mM in 10% DMSO) was dissolved in either Ca²⁺ or Ba²⁺ Ringer's solution at a final concentration of 100 nM and bath-applied to oocytes for 10 min, which achieves near-maximal PKC potentiation. The TPA solution was washed out before NMDA application. Acetoxymethyl ester of bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA-AM; 50 mM stock solution in DMSO) was diluted to 0.25 mM in Ca²⁺ Ringer's solution immediately before each experiment. Niflumic acid in 0.5 M stock solution in ethanol was diluted to 0.5 mM in Ca²⁺ Ringer's solution immediately before each experiment. The extent of PKC potentiation varied from batch to batch of oocytes. Therefore, comparisons are within single batches of oocytes or between batches with the same degree of potentiation of a given receptor subtype. Data represent mean $\pm$ SEM. Statistical analyses were performed by one-way ANOVA (GraphPad Prism version 3.0).

RESULTS

Extracellular Ca²⁺ affects PKC potentiation of recombinant NMDA receptors

To examine the effect of extracellular Ca²⁺ on PKC potentiation of NMDA receptors, we used oocytes expressing recombinant NMDA receptors. We applied NMDA (300 μM with 10 μM glycine) to oocytes clamped at –60 mV in Ba²⁺ or Ca²⁺ Ringer's solution before and after application of TPA. We focused on three NR1 splice variants that differ in their potentiation by PKC: NR1₀₁₁, which is prominent in forebrain and exhibits a relatively low level of potentiation by PKC; NR1₁₁₁, which is also prominent in forebrain and exhibits a moderate level of PKC potentiation; and NR1₁₀₀, which is prominent in midbrain and exhibits a high level of potentiation by PKC (Fig. 1*A*) (Durand et al., 1993). This choice of splice variants permitted us to evaluate the action of Ca²⁺ on receptors with and without the N-terminal splice cassette *N1* by comparison of NR1₀₁₁ and NR1₁₁₁ receptors and on receptors with and without the C-terminal splice cassettes *C1* and *C2* by comparison of NR1₁₀₀ and NR1₁₁₁ receptors. (When *C2* is spliced out, a new unrelated reading frame is opened, which encodes an alternative cassette, *C2'*, of 22 amino acids before a new stop codon is reached.)

In 1 mM Ca²⁺ Ringer's solution, TPA (100 nM) potentiated NMDA responses of NR1₀₁₁-, NR1₁₁₁-, and NR1₁₀₀-injected oocytes to 3.1 ± 0.2 -fold ($n = 19$), 6.2 ± 0.4 -fold ($n = 21$), and 12.2 ± 0.8 -fold ($n = 16$) of control NMDA responses, respectively (Fig. 1*A,B*). In 1 mM Ba²⁺ Ringer's solution, NMDA responses of all three splice variants lacked the initial peak current observed in the presence of extracellular Ca²⁺ (Fig. 1*A*, bottom row). In Ba²⁺, TPA potentiated NMDA responses of NR1₀₁₁-, NR1₁₁₁-, and NR1₁₀₀-injected oocytes to 2.3 ± 0.3 -fold ($n = 8$), 2.8 ± 0.3 -fold ($n = 8$), and 5.7 ± 0.4 -fold ($n = 10$) of the control responses, respectively (Fig. 1*A,B*). Thus, PKC potentiation of the NR1 splice variants containing *N1* (NR1₁₁₁ and NR1₁₀₀) was markedly greater in Ca²⁺ than in Ba²⁺, whereas potentiation of the splice variant NR1₀₁₁, which lacks *N1*, was only slightly greater in Ca²⁺ than in Ba²⁺ Ringer's solution.

We show below that the greater PKC potentiation recorded in Ca²⁺ requires a rise in Ca²⁺ at the intracellular opening of the channel during responses to NMDA after activation of PKC by TPA; we term this effect “Ca²⁺ amplification.” The magnitude of the Ca²⁺ amplification is determined by comparison with potentiation measured in Ba²⁺, which we assume to have no amplifying action. Because potentiation is measured as the ratio of responses measured in the same Ringer's solution after and before TPA application, differences in single-channel conductances in Ca²⁺ and Ba²⁺ Ringer's solution should not be a factor, except insofar as PKC potentiation may change channel permeability.

Figure 1. PKC potentiation of NR1 receptor splice variants differs when measured in Ca²⁺ and in Ba²⁺ Ringer's solution. *Xenopus* oocytes were injected with NR1₀₁₁, NR1₁₁₁, or NR1₁₀₀ RNA, alone or with NR2A RNA for expression of heteromeric receptors. Three to 7 d after RNA injection, NMDA responses were recorded from oocytes at -60 mV membrane potential using two-electrode voltage clamp. Currents were elicited by bath application of NMDA (300 μM with 10 μM glycine); arrowheads indicate the points of measurement of response amplitude. **A**, Whole-cell currents recorded before (left) and after (right) 10 min incubation with 100 nM TPA in Ca²⁺ Ringer's solution (top records) or Ba²⁺ Ringer's solution (bottom records). In Ca²⁺ Ringer's solution, splicing in the NR1 cassette and splicing out the C1 and C2 cassettes increased the degree of PKC potentiation. In Ba²⁺ Ringer's solution, splicing out the C1 and C2 cassettes increased PKC potentiation, but the NR1 cassette had little or no effect. In Ba²⁺ Ringer's solution, current amplitudes of all three splice variants lacked the initial peak current and late, slow rising phase observed in some records in Ca²⁺ Ringer's solution. Each pair of records (before and after TPA) is from a different oocyte. Arrowheads indicate levels at which currents were measured. Current calibration is 100 nA for NR1₀₁₁ responses recorded in Ca²⁺ and in Ba²⁺ and for NR1₁₁₁ responses recorded in Ba²⁺; calibration is 200 nA for all other responses. **B**, PKC potentiation of NR1₀₁₁, NR1₁₁₁, and NR1₁₀₀ recep-

tors in Ca²⁺ Ringer's solution and Ba²⁺ Ringer's solution. Potentiation was measured as the ratio of the NMDA-induced current (minimum current amplitude after the initial peak response) after TPA application (I_{TPA}) to the control current before TPA application ($I_{control}$). In Ca²⁺ Ringer's solution, TPA potentiated NMDA responses of NR1₀₁₁, NR1₁₁₁, and NR1₁₀₀ receptors to 3.1 ± 0.2-, 6.2 ± 0.4-, and 12.2 ± 0.8-fold of control responses, respectively (open bars). In Ba²⁺ Ringer's solution, TPA potentiated responses of NR1₀₁₁, NR1₁₁₁, and NR1₁₀₀ receptors to 2.3 ± 0.3-, 2.8 ± 0.3-, and 5.7 ± 0.4-fold of control responses, respectively (filled bars). The degree of potentiation of NR1₀₁₁ receptors observed in Ca²⁺ Ringer's solution was slightly greater than that observed in Ba²⁺ Ringer's solution ($p < 0.05$). In Ca²⁺, but not in Ba²⁺, PKC potentiation of NR1₁₁₁ receptors was significantly greater than that of NR1₀₁₁ receptors ($p < 0.001$; $p > 0.05$). For both NR1₁₁₁ and NR1₁₀₀ receptors, the degree of PKC potentiation in Ba²⁺ Ringer's solution was significantly less than in Ca²⁺ Ringer's solution ($p < 0.001$). In both Ca²⁺ and Ba²⁺, PKC potentiation of NR1₁₀₀ receptors was significantly greater than that of NR1₁₁₁ in the same solution ($p < 0.001$). In this and the following figures, data represent mean ± SEM; the numbers of experiments are indicated above each bar. **C**, PKC potentiation of heteromeric NR1₀₁₁/NR2A, NR1₁₁₁/NR2A, and NR1₁₀₀/NR2A receptors in Ca²⁺ Ringer's solution and Ba²⁺ Ringer's solution. Measured as in **B**. In 1 mM Ca²⁺ Ringer's solution, TPA potentiated NMDA responses of heteromeric NR1₀₁₁/NR2A, NR1₁₁₁/NR2A, and NR1₁₀₀/NR2A receptors to 3.6 ± 0.5-fold ($n = 4$), 8.2 ± 0.3-fold ($n = 4$), and 13.7 ± 1.8-fold ($n = 4$) of control NMDA responses, respectively (open bars). In 1 mM Ba²⁺ Ringer's solution, TPA potentiated NMDA responses of NR1₀₁₁/NR2A, NR1₁₁₁/NR2A, and NR1₁₀₀/NR2A receptors to 3.6 ± 0.4-fold ($n = 4$), 4.7 ± 0.6-fold ($n = 5$), and 7.0 ± 0.7-fold ($n = 5$) of the control responses, respectively (filled bars).

In Ba²⁺ Ringer's solution, potentiation of NR1₀₁₁ receptors was virtually identical to that of NR1₁₁₁ receptors and about half of that of NR1₁₀₀ receptors (Fig. 1*A,B*). Thus, PKC potentiation in Ba²⁺ is little affected by the NR1 cassette but is greater for the NR1 splice variants lacking the C-terminal splice cassettes C1 and C2 (see Durand et al., 1993).

Native NMDA receptors are likely to be heteromeric receptors containing both NR1 and NR2 subunits (Sheng et al., 1994; Behe et al., 1995; Blahos and Wenthold, 1996). To determine whether (1) alternative splicing of NR1 affects PKC potentiation of heteromeric receptors, and (2) there is Ca²⁺ amplification of their responses, similar experiments were performed on oocytes expressing NR2A and one of the three NR1 subunits, NR1₀₁₁, NR1₁₁₁, and NR1₁₀₀. In 1 mM Ca²⁺ Ringer's solution, TPA potentiated NMDA responses of heteromeric NR1₀₁₁/NR2A, NR1₁₁₁/NR2A, and NR1₁₀₀/NR2A receptors to 3.6 ± 0.5-fold ($n = 4$), 8.2 ± 0.3-fold ($n = 4$), and 13.7 ± 1.8-fold ($n = 4$) of control NMDA responses, respectively (Fig. 1*C*, open bars). In Ba²⁺ Ringer's solution, TPA potentiated NMDA responses of NR1₀₁₁/NR2A, NR1₁₁₁/NR2A, and NR1₁₀₀/NR2A receptors to 3.6 ± 0.4-fold ($n = 4$), 4.7 ± 0.6-fold ($n = 5$), and 7.0 ± 0.7-fold ($n = 5$) of the control responses, respectively (Fig. 1*C*, filled bars). These findings indicate that PKC potentiation of heteromeric NR1/NR2A receptors is affected by alternative splicing and exhibits Ca²⁺ amplification of the potentiated responses.

The dependence of PKC potentiation on the extracellular Ca²⁺ concentration was investigated over the range of 0.1–10 mM extracellular Ca²⁺ (Fig. 2). At higher extracellular Ca²⁺ concentrations (5–10 mM), the control currents of each of the NR1 splice variants (but not necessarily Ca²⁺ flux through the channel) were reduced compared with those observed at 0.1 mM Ca²⁺ (Fig. 2*A*; also see Jahr and Stevens, 1993). However, PKC potentiation was markedly increased for both receptors (Fig. 2). Much of the increase in potentiation of NR1₀₁₁ was a result of decrease in control responses rather than increase in the potentiated re-

Figure 2. The degree of PKC potentiation varies with extracellular Ca^{2+} concentration. *A*, Responses of NR1₀₁₁ and NR1₁₀₀ receptors were elicited by bath application of NMDA (300 μM with 10 μM glycine) before (left) and after (right) incubation with TPA; responses were measured at indicated concentrations of extracellular Ca^{2+} . Each pair of records is from a different oocyte. At 5 and 10 mM Ca^{2+} , NaCl was decreased to maintain Cl^- concentration (see Materials and Methods). PKC potentiation of both receptors increased with extracellular Ca^{2+} concentration in the range of 0.1–5 mM. Control responses (recorded before treatment with TPA) decreased in amplitude with extracellular Ca^{2+} . Current calibration is 100 nA for NR1₀₁₁, 200 nA for NR1₁₀₀ in 0.1 and 10 mM Ca^{2+} , and 500 nA for other NR1₁₀₀ records. *B*, PKC potentiation of NR1₀₁₁ and NR1₁₀₀ receptors as a function of extracellular Ca^{2+} concentration. Data represent mean $\pm$ SEM of responses of at least three oocytes for each point.

sponses; however, if single-channel conductance was unaffected by potentiation, open probability must have been increased at elevated Ca^{2+} . PKC-induced potentiation of NR1₁₀₀ responses was decreased at 10 mM Ca^{2+} , relative to that at 5 mM Ca^{2+} ; the reduction may reflect greater Ca^{2+} -induced inactivation. These findings suggest that Ca^{2+} amplification of PKC potentiation increases with Ca^{2+} concentration over a considerable range. Because potentiation of NR1₀₁₁ is increased by increasing external Ca^{2+} , both *NI* containing and *NI* lacking splice variants may share a common mechanism of Ca^{2+} amplification.

Ca^{2+} amplification is not a result of Ca^{2+} -activated Cl^- currents

Xenopus oocytes express endogenous chloride channels that are activated by cytoplasmic free Ca^{2+} [to generate $I_{\text{Cl}(\text{Ca})}$]. We used the Cl^- channel blocker niflumic acid to test for possible contamination of the Ca^{2+} amplification of NMDA evoked re-

sponses by $I_{\text{Cl}(\text{Ca})}$. In Figure 3*A* niflumic acid at 0.5 mM, a concentration that largely blocks $I_{\text{Cl}(\text{Ca})}$ evoked by Ca^{2+} entry mediated by ionophore or through voltage-dependent Ca^{2+} channels (Leonard and Kelso, 1990; White and Aylwin, 1990), blocked 17% of the peak (measured to baseline) and 15% of the steady-state NMDA evoked currents in oocytes expressing NR1₁₀₀/NR2A receptors at 1 mM external Ca^{2+} and -60 mV holding potential (first pair of records). Niflumic acid acted rapidly, and we saw no difference between its action whether or not it was applied 20 sec before NMDA plus niflumic acid; the action was also rapidly reversible (see White and Aylwin, 1990). After application of TPA, the same concentration of niflumic acid blocked 21% of the peak and 8% of the plateau phase of the potentiated NMDA responses (second pair of records). In another batch of oocytes, PKC potentiation was to 7.7 ± 0.4 -fold ($n = 3$) and 8.2 ± 0.5 -fold ($n = 3$) of control in the absence and presence of niflumic acid, respectively ($p > 0.05$), indicating a negligible contribution of $I_{\text{Cl}(\text{Ca})}$ to the measured PKC potentiation. The persistence of an initial peak in niflumic acid suggests that there is a contribution of receptor desensitization to this peak.

We also tested for a contribution of $I_{\text{Cl}(\text{Ca})}$ by measuring PKC potentiation near the Cl^- reversal potential, E_{Cl} . E_{Cl} is approximately -20 mV in the oocytes, as shown by reversal of a component of the early peak at this potential (Fig. 3*B*). At -12 mV, the NMDA response has three phases, an inward current through the NMDA receptors, a somewhat delayed outward current ascribable to $I_{\text{Cl}(\text{Ca})}$, and a later plateau current that is again through the NMDA receptors. The data in Figure 3*A* indicate that the plateau phase has little $I_{\text{Cl}(\text{Ca})}$ component. At -18 mV, the same components are seen, but the net current at the time of the peak in $I_{\text{Cl}(\text{Ca})}$ is inward. At potentials of -20 mV or more negative, the initial NMDA and $I_{\text{Cl}(\text{Ca})}$ peaks are not distinguishable in these records. The presence near E_{Cl} of an initial NMDA peak greater than the plateau indicates that desensitization contributes to the peak at more negative voltages, as is also indicated by the persistence of the peak in niflumic acid (Fig. 3*A*). We compared the PKC potentiation observed at -20 and -60 mV of NMDA splice variants that do and do not show Ca^{2+} amplification in 1 mM Ca^{2+} . In responses of oocytes expressing NR1₀₁₁, which do not show Ca^{2+} amplification, the degree of potentiation at -20 mV did not differ significantly from that at -60 mV (2.9 ± 0.3 at -20 mV versus 3.1 ± 0.7 at -60 mV), indicating that $I_{\text{Cl}(\text{Ca})}$ does not contribute significantly to PKC potentiation of these receptors (Fig. 3*C,D*). Responses of oocytes expressing NR1₁₁₁ and NR1₁₀₀ exhibited some reduction (35–45%) in PKC potentiation at -20 mV compared with -60 mV ($p < 0.05$ for NR1₁₁₁; $p < 0.01$ for NR1₁₀₀), which is consistent with reduced Ca^{2+} influx attributable to the reduced driving force at the more depolarized potential. PKC potentiation of NR1₁₁₁ receptors did remain greater than that of NR1₀₁₁ receptors ($p < 0.005$), although potentiation of the two receptors was essentially the same in Ba^{2+} (Fig. 1*B*). Because Ca^{2+} amplification was not abolished at -20 mV, we conclude that it is not a result of Ca^{2+} -activated Cl^- current. Although splicing in the *C1* and *C2* cassettes reduced potentiation in Ba^{2+} at -60 mV, this effect was not present at -20 mV.

Ca^{2+} amplification requires extracellular Ca^{2+} during opening of the NMDA channel, not during TPA application

To determine whether extracellular Ca^{2+} acts during TPA application and/or in a subsequent step (e.g., opening of the NMDA

Figure 3. Ca^{2+} amplification is not Ca^{2+} activated Cl^- current. *A*, The chloride channel blocker niflumic acid has little effect on PKC potentiation measured in Ca^{2+} . In an oocyte expressing NR1₁₀₀/NR2A receptors, niflumic acid (0.5 mM) reduced the peak of the NMDA response by 17% and the plateau by 15% before TPA application (first pair of records, 300 μM NMDA with 10 μM glycine). After TPA application, niflumic acid reduced the peak by 21% and the plateau by 8% (second pair of records). Potentiation with and without niflumic acid was 8.2 ± 0.5 ($n = 3$) and 7.7 ± 0.4 ($n = 3$), respectively. Lower gain after 10 min TPA application. *B*, E_{Cl} is near -20 mV. At -12 mV the NMDA response was triphasic, the first inward phase representing current through NMDA receptors, the second outward phase resulting from superposition of $I_{\text{Cl}(\text{Ca})}$, and the third again being primarily NMDA current. An initial NMDA peak clearly seen at -12 and -18 mV is not separable from $I_{\text{Cl}(\text{Ca})}$ at more negative potentials. *C*, *D*, Ca^{2+} amplification persists near E_{Cl} . *C*, Sample records showing NMDA elicited responses before and after PKC potentiation at -20 and -60 mV of NR1₀₁₁, NR1₁₁₁, and NR1₁₀₀ receptors. The plateau phase of the responses was reduced at -20 mV; the initial peak was reduced to a greater extent. Current calibrations are in the order of the pairs of records. *D*, PKC potentiation from records like those in *C*. Potentiation of NR1₀₁₁ receptors was essentially the same at -20 and -60 mV, indicating that there was no contribution from $I_{\text{Cl}(\text{Ca})}$. For both NR1₁₁₁ and NR1₁₀₀ receptors, PKC potentiation measured at -20 mV was smaller than that measured at -60 mV ($p < 0.05$ for NR1₁₁₁; $p < 0.1$ for NR1₁₀₀ receptors). The reduction in potentiation may have been a result of decrease in the driving force for Ca^{2+} . At -20 mV potentiation of NR1₁₁₁ receptors was greater than that of NR1₀₁₁ receptors ($p < 0.005$); potentiation of the two receptors was virtually identical in Ba^{2+} (Fig. 1*C*). Thus, even at -20 mV, PKC potentiation of the NR1₁₁₁ receptor shows Ca^{2+} amplification.

Figure 4. Ca^{2+} amplification requires extracellular Ca^{2+} during NMDA application, not during PKC activation by TPA. *Xenopus* oocytes were injected with RNA encoding NR1₁₀₀ (the NR1 splice variant that exhibits the greatest potentiation by PKC). A different oocyte was used to generate each panel of this figure. Currents were elicited by bath application of NMDA (300 μM with 10 μM glycine) in 1 mM Ca^{2+} Ringer's solution (*A*), 1 mM Ba^{2+} Ringer's solution (*C*) or the two alternately (*B*, *D*, *E*) before and after 10 min treatment with 100 nM TPA in either Ca^{2+} or Ba^{2+} Ringer's solution. The open and filled bars above the records indicate Ca^{2+} and Ba^{2+} Ringer's solution, respectively. Calibrations are the same for *A*–*E*. *A*, *B*, The presence of Ca^{2+} during incubation with TPA did not affect potentiation of NMDA responses measured in Ca^{2+} . Application of TPA in Ca^{2+} Ringer's solution potentiated the NMDA response measured in Ca^{2+} Ringer's solution to 8.3-fold of the control response measured in Ca^{2+} Ringer's solution. Application of TPA in Ba^{2+} Ringer's solution potentiated the response measured in Ca^{2+} to 7.7-fold of the control response measured in Ca^{2+} Ringer's solution. *C*, *D*, The presence of Ca^{2+} during TPA incubation had little effect on potentiation of NMDA responses measured in Ba^{2+} Ringer's solution. Treatment of a representative oocyte with TPA in Ba^{2+} Ringer's solution potentiated the NMDA response measured in Ba^{2+} to 5.2-fold of the control response measured in Ba^{2+} ; treatment with TPA in Ca^{2+} Ringer's solution potentiated the NMDA response measured in Ba^{2+} by 4.3-fold. Similar results were obtained in three independent experiments involving different batches of oocytes. *E*, NMDA responses from a single oocyte recorded in Ca^{2+} and Ba^{2+} Ringer's solution before (left records) and after (right records) application of TPA in Ba^{2+} Ringer's solution. The potentiation measured as the ratio of the responses in Ca^{2+} Ringer's solution (14-fold) was greater than that for the responses in Ba^{2+} Ringer's solution (6-fold) and was similar to that observed when the entire sequence was performed in Ca^{2+} Ringer's solution. *F*, NMDA responses of an oocyte expressing NR1₁₀₀ receptors recorded in 1 mM Ba^{2+} , then 1 mM Ca^{2+} , then 1 mM Ba^{2+} again, after application of TPA in 1 mM Ba^{2+} . On changing from Ba^{2+} to Ca^{2+} Ringer's solution, the response rose immediately from the response in Ba^{2+} to a peak and then fell to a plateau; the time course and amplitude of the response in Ca^{2+} was similar to that observed when NMDA was applied in Ca^{2+} without previous application in Ba^{2+} . On transfer to Ba^{2+} solution, the NMDA response immediately decayed to near the level of the potentiated response observed in Ba^{2+} .

channel), we substituted 1 mM Ba^{2+} for 1 mM Ca^{2+} during (1) TPA application; (2) both TPA and NMDA application; and (3) NMDA application only. For the oocytes illustrated in Figure 4, *A* and *B*, application of TPA in Ba^{2+} Ringer's solution potentiated the NMDA response measured in Ca^{2+} Ringer's solution (Fig. 4*B*) to a level similar to that observed when TPA was applied in Ca^{2+} Ringer's solution (Fig. 4*A*; to 7.7 and 8.3 times

control, respectively). In Figure 4, *C* and *D*, application of TPA in Ba^{2+} Ringer's solution potentiated the NMDA response measured in Ba^{2+} (Fig. 4*C*) to a level similar to that observed when TPA was applied in Ca^{2+} Ringer's solution (Fig. 4*D*; to 5.2 and 4.3 times control, respectively). In Figure 4*E*, control responses were obtained in Ca^{2+} and in Ba^{2+} Ringer's solution; then TPA was applied in Ba^{2+} , and a test response was obtained. Next the oocyte was transferred to Ca^{2+} Ringer's solution, and a test response was obtained within 20 sec. The degree of potentiation measured in Ca^{2+} was greater than that for Ba^{2+} (14- vs 6-fold) and as great as it would have been if the entire experiment had been performed in Ca^{2+} . These findings indicate that the Ca^{2+} amplification requires Ca^{2+} during the response to NMDA rather than during the action of TPA.

To examine the time course of onset and reversal of Ca^{2+} amplification, we first treated an oocyte expressing NR1₁₀₀ receptors with TPA in 1 mM Ba^{2+} Ringer's solution. We then applied NMDA in 1 mM Ba^{2+} , changed the solution to NMDA in 1 mM Ca^{2+} , and then returned to NMDA in Ba^{2+} (Fig. 4*F*). Transfer of the oocyte from Ba^{2+} to Ca^{2+} resulted in a rapid rise to the potentiation level observed in Ca^{2+} ; the response was characterized by a peak, followed by a decay to a plateau value. Transfer of the oocyte from Ca^{2+} to Ba^{2+} resulted in a rapid return to near the potentiated level in Ba^{2+} . These findings suggest that Ca^{2+} amplifies the NMDA response, and that onset and decay of the Ca^{2+} amplification are too rapid to be resolved within the limitations of perfusion in the oocyte system.

Ca^{2+} amplification requires a rise in intracellular free Ca^{2+}

To test whether a rise in intracellular Ca^{2+} is required for Ca^{2+} amplification of NMDA responses, we measured TPA potentiation with and without pretreatment of oocytes with the membrane-permeant Ca^{2+} chelator BAPTA-AM. In Figure 5*A*, PKC potentiation was to 12.5 times control in Ca^{2+} Ringer's solution and to 6.8 times control in Ba^{2+} Ringer's solution. Pretreatment with BAPTA-AM for 20 min had little effect on steady-state currents in Ca^{2+} or in Ba^{2+} (although it did reduce the initial peak in Ca^{2+}), but after BAPTA, PKC potentiation of NR1₁₀₀ receptors measured in Ca^{2+} was reduced to 7.8 times control, a value near that obtained in Ba^{2+} (6.4 times control; Fig. 5*B*). To determine whether BAPTA-AM inhibits activation of PKC by TPA, BAPTA-AM was applied to another oocyte after treatment with TPA (Fig. 5*C*). Under these conditions, PKC potentiation was similar in Ca^{2+} and in Ba^{2+} , i.e., to 5.5 and 5.7 times control, respectively, and was close to that in Ba^{2+} without BAPTA treatment (Fig. 5*A*). In an additional batch of oocytes expressing NR1₁₀₀, we compared potentiation in controls and after BAPTA treatment, as in Figure 5, *A* and *B*. BAPTA treatment reduced potentiation measured in Ca^{2+} to a level not significantly different from that in Ba^{2+} without BAPTA (Fig. 5*D*). Thus, BAPTA treatment blocks Ca^{2+} amplification. BAPTA also reduced PKC potentiation measured in Ba^{2+} compared with the control potentiation measured in Ba^{2+} , but the effect was not significant ($p > 0.05$). These findings indicate that this degree of BAPTA treatment reduces or blocks Ca^{2+} amplification of the PKC-potentiated response but has little effect on PKC action or on the response measured in Ba^{2+} . Longer BAPTA treatment reduced potentiation in Ba^{2+} to a greater extent, which may reflect a requirement for cytoplasmic Ca^{2+} in TPA action.

Figure 5. Intracellular BAPTA eliminates Ca^{2+} amplification. *Xenopus* oocytes from the same batch were injected with NR1₁₀₀ RNA. NMDA (300 μM with 10 μM glycine) was applied in Ca^{2+} or Ba^{2+} Ringer's solution. *A*, In this oocyte, NMDA-induced currents were potentiated to 12.5-fold of control when measured in Ca^{2+} Ringer's solution and 6.8-fold of control when measured in Ba^{2+} Ringer's solution. The potentiated response in Ca^{2+} showed a late, slowly rising phase (see Discussion). The point at which response amplitude was measured for calculation of potentiation is indicated by an arrowhead. *B*, A different oocyte was incubated in 0.25 mM BAPTA-AM for 20 min after NMDA applications in Ca^{2+} and in Ba^{2+} Ringer's solution. Responses in Ca^{2+} and in Ba^{2+} Ringer's solution were little affected by BAPTA-AM application. Subsequent application of TPA (in Ca^{2+} Ringer's solution) potentiated the responses to 6.4-fold in Ba^{2+} and 7.8-fold in Ca^{2+} ; i.e., BAPTA had little effect on PKC potentiation measured in Ba^{2+} but reduced the potentiation measured in Ca^{2+} to near the level measured in Ba^{2+} . *C*, A third oocyte was incubated in 0.25 mM BAPTA-AM for 20 min after 10 min in 100 nM TPA. PKC potentiation measured in Ca^{2+} Ringer's solution was reduced to 5.5-fold of control, similar to that measured in Ba^{2+} Ringer's solution (5.7-fold of control). *D*, Oocytes from a single batch were tested with NMDA in Ca^{2+} and in Ba^{2+} and then treated with TPA or with BAPTA followed by TPA and again tested with NMDA in Ca^{2+} and in Ba^{2+} as in *A* and *B*, respectively. PKC potentiation measured in Ca^{2+} was lower in oocytes treated with BAPTA than in controls ($p < 0.001$, ANOVA) and slightly but not significantly lower than in controls measured in Ba^{2+} . Potentiation measured in Ba^{2+} was lower in oocytes treated with BAPTA than in controls, but not significantly so ($p > 0.05$). Potentiation of BAPTA-treated oocytes measured in Ca^{2+} and in Ba^{2+} was not significantly different ($p > 0.05$).

Figure 6. Mutant NMDA receptors with reduced Ca^{2+} permeability do not exhibit Ca^{2+} amplification. **A**, Schematic of the NR1 receptor subunit with the amino acid sequence of the channel-lining domain (M2). The substitution N598R in NR1₀₁₁ (and N619R for the N1-containing receptors) greatly reduces their Ca^{2+} permeability. **B**, Responses of *Xenopus* oocytes injected with NR1₀₁₁(N598R), NR1₁₁₁(N619R), and NR1₁₀₀(N619R) RNA. Currents were elicited by bath application of NMDA (300 μ M with 10 μ M glycine) in Ca^{2+} Ringer's solution before (left) and after (right) incubation with TPA. Responses of mutant receptors were reduced in amplitude, relative to those of the corresponding wild-type receptors, and lacked the initial peak observed in Ca^{2+} Ringer's solution. **C**, PKC potentiation of responses of the three mutant receptors. PKC potentiated NR1₀₁₁(N598R), NR1₁₁₁(N619R), and NR1₁₀₀(N619R) receptors to 2.6 ± 0.4 -, 3.2 ± 0.4 -, and 5.1 ± 0.5 -fold of the control responses, respectively. PKC potentiation of the three mutant receptors measured in Ca^{2+} Ringer's solution did not differ significantly from that of the corresponding wild-type receptor measured in Ba^{2+} Ringer's solution (Ba^{2+} data from Fig. 1B; $p > 0.05$ for all three comparisons). Similarly, PKC potentiation of NR1₀₁₁(N598R) receptors and NR1₁₁₁(N619R) receptors did not differ significantly ($p > 0.05$). PKC potentiation of NR1₁₀₀(N619R) receptors was significantly greater than potentiation of the other two mutant receptors ($p < 0.05$).

NMDA receptors with reduced Ca^{2+} permeability do not exhibit the Ca^{2+} amplification

To characterize further the effect of Ca^{2+} on PKC-potentiated responses, we engineered the mutations N598R (for N1-lacking receptors) and N619R (for N1-containing receptors) in the M2 (channel lining or P) region of each of the three NR1 splice variants (Fig. 6A). Introduction of an Arg in place of N598 greatly reduces Ca^{2+} permeability of recombinant NMDA receptors (Burnashev et al., 1992; Kawajiri and Dingledine, 1993; Sakurada et al., 1993). In Ca^{2+} Ringer's solution, current amplitudes in oocytes expressing the NMDA receptor channel mutant were

Figure 7. PKC potentiation does not vary with agonist concentration. Responses of NR1₀₁₁ and NR1₁₀₀ receptors elicited by 5 μ M or 300 μ M NMDA with 10 μ M glycine were recorded in 1 mM Ca^{2+} Ringer's solution before and after TPA application. For both receptors, TPA potentiation of responses elicited by 5 μ M NMDA (open bars) did not differ significantly from potentiation of responses elicited by 300 μ M NMDA (cross-hatched bars; $p > 0.05$). For NR1₀₁₁ receptors, control responses at 5 μ M NMDA were reduced to $55 \pm 8\%$ of control responses at 300 μ M NMDA and for NR1₁₀₀ receptors to $19 \pm 4\%$ of control responses at 300 μ M NMDA.

reduced compared with those of wild-type receptors and lacked the initial peak (Fig. 6B; currents were measured in oocytes from the same batch injected with the same amount of RNA). PKC potentiation of the mutant NR1₀₁₁(N598R) receptors did not differ significantly from that of wild-type NR1₀₁₁ receptors (2.6 ± 0.4 - vs 3.1 ± 0.2 -fold in Ca^{2+} Ringer's solution; Fig. 6C, wild-type data from Fig. 1B). However, PKC potentiation of NR1₁₁₁(N619R) receptors was markedly reduced relative to that of the corresponding wild-type receptor (3.2 ± 0.4 - vs 6.2 ± 0.4 -fold). PKC potentiation of NR1₁₀₀(N619R) receptors was also reduced relative to that of the corresponding wild-type receptor (5.1 ± 0.5 - vs 12.2 ± 0.8 -fold). In all three cases, PKC potentiation of the mutant NMDA receptor measured in Ca^{2+} Ringer's solution was similar to that of the corresponding wild-type receptor measured in Ba^{2+} Ringer's solution (Fig. 6C). Thus, the M2 channel mutation abolished the Ca^{2+} amplification.

PKC potentiation and Ca^{2+} amplification are independent of agonist concentration

To investigate further whether PKC potentiation and Ca^{2+} amplification are reduced under conditions of reduced Ca^{2+} influx, we compared PKC potentiation of NR1₀₁₁ and NR1₁₀₀ receptors at 5 and 300 μ M NMDA (with 10 μ M glycine). For NR1₀₁₁ receptors, the current amplitude at 5 μ M NMDA was $55 \pm 8\%$ of that at 300 μ M NMDA; for NR1₁₀₀ receptors, the current amplitude at 5 μ M NMDA was $19 \pm 4\%$ of that at 300 μ M NMDA. The degree of PKC potentiation, however, was similar at the two agonist concentrations (4.4 ± 0.4 at 5 μ M NMDA vs 3.7 ± 0.3 at 300 μ M NMDA for NR1₀₁₁ receptors; 9.8 ± 1.9 at 5 μ M NMDA vs 8.7 ± 0.4 at 300 μ M NMDA for NR1₁₀₀ receptors) (Fig. 7). The finding that PKC potentiation is unchanged at low agonist concentration suggests that Ca^{2+} amplification is the same at low and high mean current levels per channel. It follows that Ca^{2+} amplification requires neither spatial summation of Ca^{2+} influx from

neighboring channels nor temporal summation of Ca^{2+} influx from successive periods when the receptor is fully occupied by agonists during NMDA and glycine application. (Temporal summation might occur between successive openings in a burst.) Ca^{2+} inactivation of NMDA receptors also occurs at low agonist concentration (Legendre et al., 1993) and thus also appears not to require summation of Ca^{2+} influx from successive binding events or neighboring channels.

Neutralization of positively charged residues within the N1 splice cassette reduces PKC potentiation of NMDA receptors

The N1 splice cassette alters agonist affinity, current amplitude, spermine and Zn^{2+} potentiation, and proton inhibition of recombinant NMDA receptors (Durand et al., 1992, 1993; Hollmann et al., 1993; Zhang et al., 1994; Zheng et al., 1994; Traynelis et al., 1995), and many of these effects are reversed by neutralization of positive charges in N1. To determine whether the greater PKC potentiation observed for splice variants containing the N1 cassette is affected by the presence of the six positively charged residues, we substituted the neutral amino acid alanine for each of three positively charged residues at either end of the N1 splice cassette (N1-1 and N1-2) or for all six positively charged residues (N1-3; Fig. 8A). The three mutant receptors exhibited reduced current amplitudes relative to that of the wild-type NR1₁₁₁ receptor (data not illustrated). In Ca^{2+} Ringer's solution, PKC potentiation of N1-1, N1-2, and N1-3 receptors was reduced markedly relative to that of wild-type NR1₁₁₁ receptors (to 3.5 ± 0.4 -, 2.9 ± 0.3 -, and 4.1 ± 0.5 -fold of control responses, respectively) and was similar to potentiation of wild-type NR1₀₁₁ receptors (3.1 ± 0.2 -fold; Fig. 8B). Because neutralization of the positive charges in the N1 insert reduces current amplitude (defined as the NMDA response obtained after injection of a constant amount of RNA) (Hollmann et al., 1993; Zheng et al., 1994), reduction in Ca^{2+} influx could account for the observed decrease in PKC potentiation.

DISCUSSION

PKC potentiation of recombinant NMDA receptors is amplified by Ca^{2+} influx

In the present study, alternative splicing and extracellular Ca^{2+} were shown to affect PKC potentiation of recombinant NR1 receptors expressed in *Xenopus* oocytes. For N1-containing receptors, PKC potentiation was greater when measured in Ca^{2+} Ringer's solution than when measured in Ba^{2+} Ringer's solution. The data are consistent with a mechanism in which Ca^{2+} entering through the activated receptor binds to the channel or a closely associated molecule to increase channel opening; this amplification would be greater after PKC action. Contamination by Ca^{2+} -activated Cl^- currents was minimal as discussed below. Ca^{2+} was effective when present during the application of NMDA and had no effect during activation of PKC by TPA. The evidence that the amplification requires a rise in intracellular Ca^{2+} is the following: (1) amplification is increased by increasing extracellular Ca^{2+} (Fig. 2); (2) amplification is reduced by depolarizing to reduce the driving force for Ca^{2+} (Fig. 3); (3) amplification is reduced in mutant receptors with reduced Ca^{2+} permeability (Fig. 6); and (4) amplification is prevented by applying a membrane-permeant form of the Ca^{2+} chelator BAPTA (Fig. 5). In addition, the splice variants containing the N1 cassette exhibit greater Ca^{2+} amplification (Fig. 1), possibly because they generate larger currents, which could be associated with greater

Figure 8. Neutralization of positive charges within N1 reduces Ca^{2+} amplification. **A**, NR1 receptor subunit with predicted membrane domains and N1 cassette sequence alignment for the wild-type NR1₁₁₁ receptor and the three mutant receptors, N1-1, N1-2, and N1-3. Substitutions of alanine for positively charged amino acids within the N1 insert are indicated in bold. **B**, PKC potentiation of wild-type NR1₀₁₁, mutant N1-1, N1-2, and N1-3, and wild-type NR1₁₁₁ receptors. Experiments were performed in Ca^{2+} Ringer's solution. PKC potentiated responses of NR1₁₁₁(N1-1), NR1₁₁₁(N1-2), and NR1₁₁₁(N1-3) receptors to 3.5 ± 0.4 -, 2.9 ± 0.3 -, and 4.1 ± 0.5 -fold of control responses. These values were similar to those for wild-type NR1₀₁₁ receptors (3.1 ± 0.2 ; Fig. 1B; $p > 0.05$ for N1-1 and N1-2; $p < 0.05$ for N1-3) and significantly less than the value for wild-type NR1₁₁₁ receptors (6.0 ± 0.4 ; $p < 0.005$).

Ca^{2+} influx. The reduced amplification observed after neutralization of positive charges in the N1 cassette (Fig. 8) is consistent with this interpretation, because these mutations reduce current amplitude. Furthermore, the N1-lacking receptor, NR1₀₁₁, which has a smaller current amplitude, exhibited Ca^{2+} amplification at increased extracellular Ca^{2+} ; thus, the N1 cassette is not required for Ca^{2+} amplification.

The degree of Ca^{2+} amplification was evaluated by comparison with responses in Ba^{2+} . Ba^{2+} is unlikely to have any amplifying effect, because potentiation in Ba^{2+} was similar to that in Ca^{2+} after BAPTA (Fig. 5) and to that of the mutant receptor with reduced Ca^{2+} permeability (Fig. 6). In the simplest model, PKC does not affect the Ca^{2+} amplification directly but increases channel open time and/or conductance, thereby increasing Ca^{2+} influx and the amount of amplification. PKC may also increase sensitivity of the receptor to intracellular Ca^{2+} . Alternatively, PKC action may increase single-channel conductance by reducing divalent ion binding in the channel (as has been suggested for PKC action on Mg^{2+} block of NMDA receptors in trigeminal neurons; Chen and Huang, 1992); if Ca^{2+} ions were affected more than Ba^{2+} ions, it could account for the greater potentiation in Ca^{2+} . Because BAPTA blocks the Ca^{2+} amplification after

PKC, it is unlikely that PKC increases channel conductance. Another possibility might be that PKC increases the plateau response by reducing Ca^{2+} -dependent inactivation. This mechanism is unlikely, because the action of BAPTA indicates that there is little Ca^{2+} -dependent inactivation either before or after TPA application. (Moreover, the inactivation would have to be rapid enough to be undetected in the rising phase of the response to bath applied agonists.) A final possibility to be considered is that Ca^{2+} causes insertion of new receptor molecules. This mechanism also appears unlikely because of the rapid onset and reversibility of the Ca^{2+} amplification (Fig. 4).

Ca^{2+} amplification is not an artifact of Ca^{2+} -activated chloride current

A number of observations indicate that $I_{\text{Cl}(\text{Ca})}$ does not contribute significantly to PKC potentiation or Ca^{2+} amplification, as measured in this study. (1) The chloride channel blocker, niflumic acid, had only a small effect on the amplitude of the plateau responses and did not reduce PKC potentiation measured in 1 mM Ca^{2+} Ringer's solution at -60 mV (Fig. 3A). Leonard and Kelso (1990) reported a greater effect of niflumic acid on NMDA responses in oocytes expressing rat brain mRNA, but this difference may have resulted from use of brain mRNA, which may encode additional Ca^{2+} -activated Cl^- channels and from recording at -80 mV in 2 mM external Ca^{2+} , which would increase $I_{\text{Cl}(\text{Ca})}$. (2) Ca^{2+} amplification is present at -20 mV, a value near E_{Cl} in oocytes (Fig. 3B,C). At this holding potential, the chloride current is small, but Ca^{2+} amplification persists, although reduced, presumably because of the reduced driving force for Ca^{2+} influx. (3) We measure in oocytes a reversal potential for recombinant NMDA receptors of about -5 mV (data not illustrated; also see Hollmann et al., 1993). This value is close to that reported for neuronal NMDA receptors (cf. Mayer and Westbrook, 1987), which indicates that there is little $I_{\text{Cl}(\text{Ca})}$ contribution. (4) The degree of Ca^{2+} amplification was not changed when Ca^{2+} influx was reduced by reducing agonist concentration (Fig. 7). Also, the degree of Ca^{2+} amplification was the same for oocytes generating large and small currents in response to saturating NMDA, reflecting differences in the level of receptor expression (data not illustrated). $I_{(\text{Ca})\text{Cl}}$ would be expected to decrease with decrease in NMDA elicited currents.

Are Ca^{2+} influx and Ca^{2+} amplification during NMDA application regenerative?

The mechanism of the proposed Ca^{2+} amplification is as yet unknown, but single-channel studies and mutational analysis will undoubtedly further elucidate the molecular basis. Ca^{2+} may act directly on the channel protein or on a closely associated accessory protein expressed by the *Xenopus* oocyte. Candidate proteins are calmodulin and Ca^{2+} - and calmodulin-dependent kinases, implicated in NMDA-dependent LTP. The rapid rise in current amplitude on transfer of the oocyte from Ba^{2+} to Ca^{2+} Ringer's solution and rapid decay on transfer from Ca^{2+} to Ba^{2+} Ringer's solution argues against a covalent modification, such as phosphorylation or dephosphorylation and in favor of a direct action of Ca^{2+} (or Ca^{2+} -calmodulin complex) on the cytoplasmic face of the receptor. Ca^{2+} -calmodulin binds to a site in the *C1* cassette and a site in *C0*, the region between TM4 and *C1* of the NR1 subunit. However, binding to these sites decreases channel open time in heteromeric receptors with NR2A expressed in HEK 293 cells (Ehlers et al., 1996).

The Ca^{2+} amplification should tend to be regenerative. However, in our experiments the amplification was independent of agonist concentration (Fig. 7). This finding, together with our observation that Ca^{2+} amplification is independent of the level of receptor expression (data not shown), suggests that there was neither spatial summation of Ca^{2+} influx from neighboring channels nor temporal summation of Ca^{2+} influx from successive bursts of openings caused by periods of full occupancy of the receptor by NMDA and glycine. It follows that under these conditions there was no regenerative response.

As noted above, neuronal receptors can show Ca^{2+} -dependent inactivation (Legendre et al., 1993; Rosenmund et al., 1995). In recombinant receptors expressed in HEK 293 cells, Ca^{2+} -dependent inactivation is affected by the NR2 subunit, being greatest in NR1/NR2A receptors, less in NR1/NR2B receptors, and virtually absent in NR1/NR2C receptors (Krupp et al., 1996). In *Xenopus* oocytes, increase in extracellular Ca^{2+} was reported to increase NMDA response amplitude comparable to the Ca^{2+} amplification proposed here; however, unlike the results in Figure 2, the response amplitude was not reduced at higher concentrations of Ca^{2+} (Koltchine et al., 1996).

Recombinant NMDA receptors expressed in *Xenopus* oocytes exhibit a late, slowly rising phase in NMDA responses, particularly evident in *N1*-containing NR1 receptors, in the presence or absence of activators of PKC (Figs. 1A, 5) (Koltchine et al., 1996). This slow, Ca^{2+} -dependent response is more likely to occur at higher agonist concentrations and after PKC potentiation. It may represent the release of Ca^{2+} from intracellular pools, although the persistence of the response in BAPTA argues against this mechanism.

Does PKC potentiation involve phosphorylation of the NR1 protein?

PKC phosphorylates specific serine residues in the NR1₀₁₁ receptor expressed transiently in HEK 293 cells (Tingley et al., 1993). The four identified series are contained in the *C1* cassette, although our electrophysiological findings indicate that splicing out the *C1* and *C2* cassettes increases PKC potentiation (Fig. 1) (Durand et al., 1992, 1993). Thus, PKC potentiation may not require phosphorylation of the receptor itself, and phosphorylation of *C1* may even reduce PKC potentiation. In agreement, mutation of the phosphorylatable serines to alanines increases TPA potentiation (Zhang et al., 1996). It is then reasonable to suggest that there is another molecule associated with NR1, phosphorylation of which is responsible for the potentiation. An obvious possibility is NR2A and/or B, which apparently are phosphorylated by PKC (Hall and Soderling, 1997; Leonard and Hell, 1997).

Functional significance of the Ca^{2+} amplification

Although our experiments were performed on recombinant NMDA receptors expressed in *Xenopus* oocytes, Ca^{2+} amplification is likely to occur after PKC potentiation of neuronal NMDA receptors. As noted above, PKC potentiates NMDA receptors in neurons from hippocampus (Aniksztejn et al., 1992), trigeminal nucleus (Chen and Huang, 1992), and spinal cord (Gerber et al., 1989). In our study, extracellular Ca^{2+} and alternative splicing were shown to affect PKC potentiation. These results together with the finding that expression of NR1 splice variants is temporally and spatially regulated (Laurie and Seeburg, 1994; Zukin and Bennett, 1995; Paupard et al., 1997) provide a mechanism whereby NMDA signals can be

amplified in specific neuronal populations at times of enhanced synaptic activity. Thus, PKC potentiation of NMDA responses may have a significant impact on cellular events including induction of long-term potentiation and synaptogenesis. Ca^{2+} amplification, if present in neurons, may have particularly important actions in small structures such as dendritic spines, where influxes through neighboring receptor channels would be more likely to interact.

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