In CA1 Pyramidal Neurons of the Hippocampus Protein Kinase C Regulates Calcium-Dependent Inactivation of NMDA Receptors

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The NMDA subtype of the glutamate-gated channel exhibits a high permeability to Ca^{2+} . The influx of Ca^{2+} through NMDA channels is limited by a rapid and Ca^{2+} /calmodulin (CaM)-dependent inactivation that results from a competitive displacement of cytoskeleton-binding proteins from the NR1 subunit of the receptor by Ca^{2+} /CaM (Zhang et al., 1998; Krupp et al., 1999). The C terminal of this subunit can be phosphorylated by protein kinase C (PKC) (Tingley et al., 1993). The present study sought to investigate whether PKC regulates Ca^{2+} -dependent inactivation of the NMDA channel in hippocampal neurons. Activation of endogenous PKC by 4 β -phorbol 12-myristate 13-acetate enhanced peak (I_p) and depressed steady-state (I_{ss}) NMDA-evoked currents, resulting in a reduction in the ratio of these currents (I_{ss}/I_p). We demonstrated previously that PKC activity enhances I_p via a sequential activation of the focal adhesion kinase cell adhesion kinase β /proline-rich tyrosine kinase 2 (CAK β /Pyk2) and the nonreceptor

tyrosine kinase Src (Huang et al., 1999; Lu et al., 1999). Here, we report that the PKC-induced depression of I_{ss} is unrelated to the PKC/CAK β /Src-signaling pathway but depends on the concentration of extracellular Ca^{2+} . Intracellular applications of CaM reduced I_{ss}/I_p and occluded the Ca^{2+} -dependent effect of phorbol esters on I_{ss} . Moreover, increasing the concentration of intracellular Ca^{2+} buffer or intracellular application of the inhibitory CaM-binding peptide (KY9) greatly reduced the phorbol ester-induced depression of I_{ss} . Taken together, these results suggest that PKC enhances Ca^{2+} /CaM-dependent inactivation of the NMDA channel, most likely because of a phosphorylation-dependent regulation of interactions between receptor subunits, CaM, and other postsynaptic density proteins.

Key words: NMDA receptor; desensitization; Ca^{2+} -dependent inactivation; calmodulin; PKC; phosphorylation; hippocampal neurons

NMDA receptors constitute a major class of the glutamate-gated ion channels that play a key role in synaptic development and plasticity in the CNS. Via their interactions with specific anchoring proteins, NMDA channels are clustered at appropriate positions within the postsynaptic density (PSD) where the regulatory protein calmodulin (CaM) can interact with the channel subunits to modulate channel gating (Ehlers et al., 1996a,b; Zhang et al., 1998; Krupp et al., 1999). The molecular organization of the PSD is essential for the precise localization of NMDA channels and is also important for the efficient transduction of intracellular signaling events that can be triggered after NMDA receptor activation (Swope et al., 1999).

NMDA receptors are oligomeric complexes comprised of three families of subunits: NR1, NR2A–D, and the newly identified NR3 subunit (Moriyoshi et al., 1991; Monyer et al., 1992; Yamakura and Shimoji, 1999). Most native NMDA receptors are thought to be heteromers composed of various combinations of NR1 and NR2 subunits (Sheng et al., 1994). Eight different splice variants of the NR1 subunits arising from alternative splicing of one short cassette in the N terminal (N1) and two cassettes in the C terminal (C1 and C2) have also been identified (Hollmann and

Heinemann, 1994; Bennett and Dingledine, 1995). All of the NR1 splice variants contain a common C0 region just proximal to the C terminal C1 and C2 cassettes (Ehlers et al., 1998). These NR1 splice variants exhibit differences in their spatial and developmental expression patterns in the CNS (Laurie and Seeburg, 1994) and their subcellular localization in heterologous expression systems (Ehlers et al., 1995), as well as their agonist and antagonist potencies (Yamakura and Shimoji, 1999).

The NR1 subunit is directly phosphorylated by PKC (Tingley et al., 1993), and activation of this kinase enhances NMDA-evoked currents in isolated trigeminal neurons (Chen and Huang, 1992) and in isolated and cultured hippocampal neurons (Xiong et al., 1998), as well as in *Xenopus* oocytes injected previously with either brain mRNA (Kelso et al., 1992) or cDNAs for recombinant NMDA receptor subunits (Zukin and Bennett, 1995). Further analysis has shown that the PKC-induced phosphorylation occurs primarily but not exclusively at four serine residues located in the C1 cassette of the NR1 subunit (Tingley et al., 1993). Interestingly, recombinant receptors lacking the C1 cassette demonstrate a greater phorbol ester-induced potentiation than do those containing this cassette (Durand et al., 1993), suggesting that the potentiation by PKC is unrelated to phosphorylation of these residues (Yamakura et al., 1993). In this regard, we have demonstrated recently that phorbol esters enhance NMDA-evoked currents in CA1 pyramidal neurons via a sequential activation of PKC, the focal adhesion kinase cell adhesion kinase β /proline-rich tyrosine kinase 2 (CAK β /Pyk2) (Huang et al., 1999), and the nonreceptor tyrosine kinase Src rather than as a consequence of the direct phosphorylation of NMDA receptors by PKC (Lu et al., 1999).

Received Jan. 6, 2000; revised April 4, 2000; accepted April 7, 2000.

The present study was supported by the Medical Research Council (MRC) of Canada. W.-Y.L. is a fellow of the Heart and Stroke Foundation of Canada, M.F.J. is a fellow of the Natural Sciences and Engineering Research Council of Canada, and D.B. is a fellow of the MRC of Canada. We thank Dr. K.-W. Yau for providing CaM-binding peptide (KY9) and its control peptide (KY8), Dr. Terukatsu Sasaki for providing recombinant CAK β /Pyk2, and Dr. M. W. Salter for Src(40–58).

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NMDA channels exhibit a high permeability to Ca^{2+} relative to most AMPA channels, and this Ca^{2+} signal underlies much of the NMDA receptor-dependent plasticity and neurotoxicity in the CNS. However, the influx is limited by a rapid Ca^{2+} - and CaM-dependent channel inactivation (Mayer and Westbrook, 1985; MacDermott et al., 1986; Rosenmund and Westbrook, 1993; Rosenmund et al., 1995). Additionally, the influx of Ca^{2+} may activate the phosphatase calcineurin resulting in a downregulation of NMDA channels (Lieberman and Mody, 1994) and an increased expression of glycine-insensitive desensitization of NMDA receptors (Tong and Jahr, 1994). In turn, the potential dephosphorylation of NMDA receptors may be counterbalanced by the serine–threonine kinases cAMP-dependent kinase (PKA) (Raman et al., 1996) or casein kinase-II (Lieberman and Mody, 1999).

Recent studies have shown that the Ca^{2+} - and CaM-dependent negative feedback requires regulation of protein–protein interactions involving NMDA receptor subunits themselves. Specifically, the C0 region of the NR1 subunit competitively binds the actin-associated protein α -actinin2 (Wyszynski et al., 1997) as well as CaM (Ehlers et al., 1996b). Inactivation may occur as a consequence of a competitive displacement of α -actinin2 by Ca^{2+} /CaM or alternatively by a Ca^{2+} -dependent reduction in the affinity of α -actinin2 for this site on the receptor (Zhang et al., 1998; Krupp et al., 1999). The phosphorylation of serine residues in the C1 cassette is also correlated with dispersion of surface-associated clusters of NR1 subunits expressed in fibroblasts (Ehlers et al., 1995, 1996a). Furthermore, the binding of the cytoskeletal protein spectrin to the C terminal of the NR1 receptor is also inhibited by PKC-dependent phosphorylation of this subunit (Wechsler and Teichberg, 1998). Therefore the present experiments examine the hypothesis that PKC-dependent phosphorylation directly regulates Ca^{2+} -dependent inactivation of NMDA receptors.

MATERIALS AND METHODS

Isolated neurons and whole-cell recordings of NMDA-evoked currents. CA1 neurons were isolated from hippocampal slices taken from postnatal rats (Wistar; 12–20 d old) using previously described procedures (Wang and MacDonald, 1995). Briefly, the rats were anesthetized with halothane and killed by decapitation using a guillotine. The whole brain was removed and placed in cold (4°C) extracellular solution (see below for composition). Hippocampi were then microdissected and cut by hand into 400- to 500- μm -thick slices using a razor blade. The slices were incubated at room temperature for 30 min in extracellular solution containing 0.3–0.5 mg/ml papain (from papaya latex; Sigma, St. Louis, MO). The slices were then washed and kept in enzyme-free solution until used. All solutions were bubbled with 100% O_2 . One slice was transferred into a 35 mm culture dish to facilitate isolation of neurons. The CA1 region was excised from the slice, and two polished glass pipettes were used to isolate single cells mechanically. Only neurons that retained their pyramidal shape, including a major primary and several secondary dendritic processes, were used for recordings. Recordings from control and drug-treated cells were always made on the same day.

The extracellular solution was composed of 140 mM NaCl, 1.3 mM CaCl_2 , 5.0 mM KCl, 25 mM HEPES, 33 mM glucose, 0.0005 mM TTX, and 3–10 μM glycine, with a pH of 7.4 and osmolarity between 325 and 335 mOsm. In some experiments the extracellular concentration of CaCl_2 was changed as indicated. In addition, 10 μM EDTA was occasionally added in the extracellular solution to chelate contaminating concentrations of Zn^{2+} . Whole-cell patch-clamp recordings were performed at room temperature (20–22°C). Neurons were voltage-clamped and lifted into the stream of the extracellular solution supplied by a computer-controlled multibarreled perfusion system (Warner). Currents were evoked by rapid application of NMDA (300 μM ; unless otherwise indicated, τ of exchange was ~ 2 msec). The resistance of patch pipettes used in the study was 3–4 M Ω . To monitor series resistance, a voltage

step of -10 mV was applied before each application of NMDA. The series resistance in the recordings was between 6 and 8 M Ω . Recordings in which series resistance varied by $>10\%$ were rejected. No electronic compensation for series resistance was used. The intracellular solution contained 140 mM CsMeSO₄ or CsF, 11 mM EGTA, 1 mM CaCl_2 , 2 mM MgCl_2 , 10 mM HEPES, 2 mM TEA, and 4 mM K_2ATP , with a pH of 7.3 and osmolarity between 295 and 300 mOsm. In certain experiments, 11 mM EGTA was replaced with 20 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) as indicated. Drugs used in this study were purchased from Sigma or as follows: 4 α -phorbol 12-myristate 13-acetate (4 α -PMA), 4 β -PMA, and chelerythrine from Alexis; pp60c-Src from Upstate Biochemicals; and Src(40–58) and sSrc(40–58) from Dr. M. W. Salter (Hospital for Sick Children, Toronto, Ontario, Canada). CaM-binding peptide (CaM inhibitory peptide, KY9) and its control peptide (KY8) were a gift from Dr. K.-W. Yau. PMA and chelerythrine were dissolved in dimethylsulfoxide (DMSO), and stock solutions were made to 10 mM concentration. All stock solutions of drugs were kept at -25 or -70°C and thawed only before the experiment. Final concentrations of DMSO used in this study were kept to $<0.01\%$.

Currents were recorded using an Axopatch 1-B amplifier, and data were filtered at 2 kHz, digitized, and acquired using the pClamp6 program (Axon Instruments). All population data are expressed as the mean $\pm$ SEM. The Student's paired *t* test or the ANOVA test was used whenever appropriate.

Nucleated patches from cultured hippocampal neurons and ultrafast perfusion. Nucleated patches (Sather et al., 1992) taken from cultured hippocampal neurons were used in conjunction with an ultrafast perfusion system. This system consisted of a Burleigh piezoelectric actuator and θ -shape perfusion tubing, the use of which allowed a more rapid delivery of the agonist. Changes of solution could be achieved within 0.4 msec.

RESULTS

Activation of PKC enhances peak and depresses steady-state NMDA-evoked currents

In acutely isolated hippocampal pyramidal neurons, rapid applications of NMDA (300 μM ; glycine, 3 μM) evoked a peak current (I_p) that rapidly desensitized or inactivated to a steady-state value (I_{ss}) (Fig. 1A). The steady-state-to-peak ratio (I_{ss}/I_p) of the current has been used previously as a measure of the degree of desensitization or inactivation of NMDA channels (Sather et al., 1992; Lu et al., 1998; Zhang et al., 1998). This ratio decreased by $\sim 8\%$ over the first 15–20 min of recording (Fig. 1B), reflecting a small and time-dependent increase in the extent of NMDA receptor desensitization (Sather et al., 1992). Among the cells examined this ratio ranged from 0.45 to 0.6 when the control intracellular solution was used.

The active phorbol ester 4 β -PMA (100 nM) was used to activate endogenous PKC. Peak currents (Fig. 1A) were enhanced by 8–35% ($12 \pm 6.6\%$; $n = 12$ cells analyzed) 5–8 min after exposure to PMA. The degree of enhancement was relatively small because of the high concentrations of NMDA and glycine used in these experiments (Lu et al., 1999). In contrast, I_{ss} was reduced by 32–71% ($51 \pm 12\%$; $n = 12$; Fig. 1D) resulting in a reduction in I_{ss}/I_p (control, $I_{ss}/I_p = 0.49 \pm 0.08$; PMA, $I_{ss}/I_p = 0.26 \pm 0.07$; $n = 12$ in both groups; Fig. 1A). The same effects were observed after applications of a different phorbol ester, phorbol 12,13-didecanoate (PDD; 1 μM ; Fig. 1A2), whereas the inactive analog of PMA, 4 α -PMA, was without effect on NMDA-activated currents (Fig. 1B,D). To confirm that the effects of PMA were caused by activation of endogenous PKC, we performed a series of recordings with or without chelerythrine, a selective inhibitor of PKC (Lu et al., 1999), in the patch pipette. Chelerythrine did not change the NMDA-evoked currents but prevented both the enhancement of I_p and the depression of I_{ss} by PMA (Fig. 1C,D).

Figure 1. Activation of endogenous PKC in hippocampal pyramidal neurons induces a complex modulation of whole-cell NMDA-evoked currents. **A1**, In an acutely isolated CA1 pyramidal neuron, currents were evoked by NMDA ($300\ \mu\text{M}$) before and during bath application (indicated by horizontal bar) of 4β -PMA ($100\ \text{nM}$). Note that the peak (I_p) increased while the steady state (I_{ss}) decreased after PMA. **A2**, The same effect is observed with applications of PDD. **Right**, Currents were normalized to the peak recorded after PDD. **B**, The inactive form of the phorbol ester 4α -PMA ($100\ \text{nM}$) was ineffective (*middle*) in contrast to 4β -PMA (*right*) applied to the same neuron. **C**, Intracellular application of the PKC inhibitor chelerythrine ($10\ \mu\text{M}$) did not affect the current itself but blocked the effect of 4β -PMA (*right*; treated with 4β -PMA for 6 min). The dashed horizontal lines in **A–C** indicate steady-state current level during control. **D**, Population data summarize changes in I_{ss}/I_p over the time course of the recording from four groups of cells. Under control conditions (gray squares) I_{ss}/I_p decreased slightly during the first 6–15 min of the recording and then stabilized ($n = 7$). Applications of 4β -PMA (black circles) reduced I_{ss}/I_p from 0.49 ± 0.08 in control to 0.26 ± 0.0751 15 min after application of PMA ($n = 12$; two-way ANOVA, $p < 0.002$). In contrast, 4α -PMA (gray triangles) had no effect ($n = 4$). The effect of 4β -PMA on I_{ss}/I_p was reduced by inclusion of chelerythrine ($10\ \mu\text{M}$; white squares) in the patch pipette ($n = 6$; two-way ANOVA, $p < 0.01$). **E**, Responses of nucleated patches to rapid applications of NMDA ($100\ \mu\text{M}$) and bath-applied PMA are shown. **E1**, Two superimposed responses of a nucleated patch taken from a cultured hippocampal neuron are shown. **E2**, 4β -PMA enhanced I_p to $131 \pm 11\%$ of control and depressed I_{ss} to $59 \pm 4\%$ of control ($n = 6$; $p < 0.05$). **E3**, This phorbol ester reduced I_{ss}/I_p from 38 ± 0.7 in control to 17 ± 0.4 ($n = 6$; $p < 0.05$).

We also examined the effects of PMA on the responses of nucleated patches to rapid applications of NMDA. This was done in case we were underestimating the actual values of I_p because of a substantial desensitization of the receptors occurring during the onset of agonist application. Confirming that this was unlikely to be the case, PMA applications to nucleated patches modified NMDA-evoked currents similarly to what was observed in whole-cell recordings (Fig. 1E).

The PKC-induced potentiation of I_p is dependent on activation of Src, but the depression of I_{ss} is not

NMDA receptor-mediated currents (I_p) in CA1 neurons are upregulated by several G-protein-coupled receptors via the sequential activation of PKC and then the tyrosine kinases $\text{CAK}\beta/\text{Pyk2}$ (Huang et al., 1999) and Src (Lu et al., 1999). Instead of a Src-dependent downregulation of I_{ss} , we hypothesized that the depression was the result of a more direct effect of PKC on NMDA channel function. To test this possibility we first perfused pp60c-Src into the patch pipette while recording NMDA-activated currents. As shown in Figure 2, A and B, this kinase enhanced I_p of the NMDA-evoked current to $131 \pm 9.0\%$ ($n = 9$) but caused no reduction in I_{ss} . Although, inclusion of Src in the patch pipette occluded the ability of PMA to potentiate I_p , it failed to prevent the depression of I_{ss} (Fig. 2A).

We demonstrated previously that intracellular applications of the Src inhibitory peptide Src(40–58) prevented both the PMA- and Src-induced potentiation of I_p in these neurons (Lu et al., 1999). However, inclusion of this peptide in the patch pipette ($25\ \mu\text{g}/\text{ml}$) failed to alter the PMA-induced depression of I_{ss} (Fig. 2C), nor did it prevent the decrease in I_{ss}/I_p (data not shown).

Similarly, intracellular applications of $\text{CAK}\beta/\text{Pyk2}$ failed to alter the PMA-induced depression of I_{ss} (Fig. 2D). Taken together, these results demonstrate that the PMA-induced depression of I_{ss} is independent of the activity of either $\text{CAK}\beta/\text{Pyk2}$ or Src.

Application of PMA enhanced the apparent desensitization of NMDA responses

The PMA-induced potentiation of I_p is relatively small in saturating concentrations of NMDA and glycine whereas the depression of I_{ss} is most prominent (Lu et al., 1999) (Fig. 3A), suggesting a potentiation of desensitization (Sather et al., 1992; Lu et al., 1998). NMDA-evoked responses demonstrate at least three different forms of desensitization or inactivation: (1) a Ca^{2+} -dependent inactivation, (2) a glycine-sensitive desensitization, and (3) a glycine-independent desensitization (McBain and Mayer, 1994). Initially, we investigated whether or not PMA had simply increased I_p , driving more of the receptors into the desensitized state and causing a relative reduction in the amplitude of I_{ss} . Using a fixed concentration of NMDA, we examined the relationship between the PMA-induced depression of I_{ss} and the potentiation of I_p . With increasing concentrations of glycine, the degree of potentiation of I_p decreased (Fig. 3A,B), and the depression of I_{ss} increased (Fig. 3A,C). The greatest depression of I_{ss} was observed in saturating concentrations of glycine [also seen in saturating concentrations of NMDA (Lu et al., 1999)]. To assess quantitatively the relationship between the degree of depression and the size of the peak currents, we plotted I_{ss} against I_p (Fig. 3D) both before and after applications of PMA. Peak and steady-state components of the evoked currents could be distinguished with glycine concentrations ranging from 300 to 3000 μM .

Figure 2. The phorbol ester-induced depression of I_{ss} was independent of Src and CAKβ/Pyk2 activity. *A*, An example of NMDA-evoked currents recorded from an isolated neuron when pp60c-Src was included in the patch pipette is shown. Times after the beginning of the whole-cell configuration are indicated above the traces. *B*, Similar recordings for a group of such cells ($n = 7$) are summarized in this plot. Inclusion of Src enhanced the peak (black circles) but did not depress steady-state (gray squares) currents. Currents in each cell were normalized to the first response 1–2 min after establishment of the whole-cell configuration. *C*, 4β-PMA depressed I_{ss} with (gray squares) or without (black circles) inclusion of the Src inhibitory peptide Src(40–58) in the patch pipette (25 μg/ml). Fifteen minutes after exposure to 4β-PMA, I_{ss} with Src(40–58) was $42 \pm 5\%$ ($n = 7$), whereas I_{ss} without Src(40–58) was $39 \pm 7\%$ ($n = 5$; $p > 0.05$). *D*, Intracellular application of CAKβ/Pyk2 (0.5 μg/ml) did not reduce I_{ss} , nor did it prevent the depression of I_{ss} by 4β-PMA.

As shown in Figure 3D the relationship between I_{ss} and I_p was linear, and the slope of this relationship (ratio of I_{ss} to I_p) was reduced by treatment of the cells with PMA (control, 0.7; PMA, 0.3; $n = 7$). This analysis suggests that the PMA-induced depression of I_{ss} was unrelated to the absolute amplitude of I_p and indicates that the reduction of I_{ss} is unlikely to have been simply a consequence of an enhanced glycine-sensitive desensitization. Nevertheless, to minimize any potential effects of PMA on

glycine-sensitive desensitization, we used saturating concentrations of glycine in most of the subsequent recordings.

Depression by PKC of I_{ss} is dependent on extracellular calcium

An alternative explanation for the PMA-induced depression of I_{ss} is an enhancement of Ca^{2+} inactivation. We therefore tested the effect of PMA on I_p and I_{ss} in the presence of three different concentrations of extracellular Ca^{2+} (0.5, 1.3, and 5.0 mM

Figure 3. The PMA-induced depression of I_{ss} was greatest in the presence of saturating concentrations of glycine. *A*, Responses of an isolated neuron to applications of various concentrations of glycine in the presence of a fixed concentration (50 μM) of NMDA are shown before (top) and after (bottom) bath application of PMA. *B*, The normalized I_p is plotted against glycine concentration before (black squares) and after (gray squares) applications of PMA ($n = 7$). Currents were normalized to the response to 10,000 nM glycine. *C*, A similar plot of I_{ss} is shown with currents normalized to that evoked by 30,000 nM glycine. Note the greater depression of the I_{ss} in the presence of higher concentrations of glycine. *D*, For the same group of recordings shown in *B* and *C*, I_{ss} was plotted against I_p before (black circles) and after (gray circles) PMA. The relationship between I_{ss} and I_p was linear. The slope of the I_{ss} – I_p relationship was reduced from 0.7 to 0.3 after treatment of the cells with PMA.

Figure 4. The PMA-induced depression of I_{ss} was dependent on extracellular calcium. **A1, B1.** In the presence of three different concentrations of extracellular Ca^{2+} (indicated above the traces), currents were evoked by a low concentration (10 μM ; **A1**) or high concentration (300 μM ; **B1**) of NMDA before (black traces) and after (gray traces) PMA. **A2.** Using the low concentration of NMDA, the slope of the I_{ss}/I_p to $[\text{Ca}^{2+}]_e$ relationship was significantly enhanced by PMA (control, black circles; slope = $-0.012 \text{ mM}^{-1} [\text{Ca}^{2+}]_e$; PMA, gray squares; slope = $-0.045 \text{ mM}^{-1} [\text{Ca}^{2+}]_e$). **B2.** Superimposed traces of the currents before and after PMA (NMDA, 300 μM) were normalized to their peaks. **C.** Potentiation of I_p by PMA was independent of $[\text{Ca}^{2+}]_e$ (0.2 mM Ca^{2+} , $26 \pm 3\%$; $n = 6$; 1.3 mM Ca^{2+} , $22 \pm 4\%$; $n = 6$; 5.0 mM Ca^{2+} , $23 \pm 4\%$; one-way ANOVA, $p = 0.25$; $n = 6$). **D.** Depression of I_{ss} was dependent on $[\text{Ca}^{2+}]_e$ (0.2 mM Ca^{2+} , control, 0.54 ± 0.06 ; PMA, 0.43 ± 0.03 ; 1.3 mM Ca^{2+} , control, 0.53 ± 0.02 ; PMA, 0.34 ± 0.03 ; 5.0 mM Ca^{2+} , control, 0.49 ± 0.08 ; PMA, 0.16 ± 0.04 ; one-way ANOVA, $p < 0.001$; $n = 6$). **E.** The slope of the I_{ss}/I_p to $[\text{Ca}^{2+}]_e$ relationship was significantly enhanced by PMA (Control, black circles; slope = $-0.021 \text{ mM}^{-1} [\text{Ca}^{2+}]_e$; PMA, gray squares; slope = $-0.055 \text{ mM}^{-1} [\text{Ca}^{2+}]_e$). Con, Cont, Control.

$[\text{Ca}^{2+}]_e$) with the objective of increasing the influx of Ca^{2+} through NMDA channels. At first we examined responses to relatively low concentrations of NMDA, mimicking previous protocols used in cultured cells (Zhang et al., 1998). Extracellular Ca^{2+} depressed the amplitude of the currents as reported previously (Ascher and Nowak, 1988; Lieberman and Mody, 1994). However, unlike cultured cells (Zhang et al., 1998) minimal time- and Ca^{2+} -dependent inactivation was observed in isolated CA1 neurons until PMA was applied. Furthermore, applications of PMA enhanced the entire response to applied NMDA (Fig. 1A1), and the steady-state current recorded in the presence of PMA was actually larger than the entire corresponding control response. The Ca^{2+} dependency of the PMA-induced inactivation was quantified by plotting I_{ss}/I_p against $[\text{Ca}^{2+}]_e$ (Fig. 4A2) and using the slope of this relationship as a measure of its Ca^{2+} sensitivity (control $k = -0.012 \text{ mM}^{-1}$; $n = 5$; PMA $k = -0.045 \text{ mM}^{-1}$; $n = 6$; two-way ANOVA, $p < 0.05$). We therefore continued to examine responses to near-saturating concentrations of NMDA in which there are smaller PMA-induced increases in I_p and large decreases in I_{ss} .

Applications of PMA proportionately enhanced I_p to a similar degree in all three tested values of $[\text{Ca}^{2+}]_e$ (Fig. 4B,C), whereas the depression of I_{ss} was proportionally greater as $[\text{Ca}^{2+}]_e$ was increased (Fig. 4B,D). The slope of the I_{ss}/I_p against $[\text{Ca}^{2+}]_e$

relationship was more than doubled after treatment of cells with PMA (before PMA, $k = -0.021 \text{ mM}^{-1} [\text{Ca}^{2+}]_e$; after PMA, $k = -0.055 \text{ mM}^{-1} [\text{Ca}^{2+}]_e$; $n = 6$; two-way ANOVA, $p < 0.05$; Fig. 4E). The enhanced Ca^{2+} -dependent inactivation occurred in spite of the greater charge transfer and influx of Ca^{2+} associated with the control responses (Fig. 4B1, 5.0 mM Ca^{2+}) compared with those after PMA treatment. This suggests that we are likely underestimating the PMA-induced enhancement of the sensitivity of inactivation because the size of the calcium signal is less after treatment with PMA than before. In one group of cells ($n = 5$) the effects of PMA on I_{ss}/I_p were also examined in the absence of added Ca^{2+} . Applications of PMA still enhanced I_p by $\sim 25\%$, but there was no depression of I_{ss} (data not shown). These results also support the hypothesis that the PMA-induced depression of I_{ss} results from an enhancement of the sensitivity of Ca^{2+} inactivation.

Buffering intracellular Ca^{2+} alters the PMA-induced depression of I_{ss}

To test the hypothesis that the PMA-induced depression of I_{ss} results from a modification of Ca^{2+} inactivation of NMDA channels, we made a series of recordings with or without the Ca^{2+} buffer EGTA (11 mM) in the patch pipette. In recordings made with EGTA, applications of PMA enhanced I_p but had little

Normalized values of I_{ss}/I_p before (black circles) and after (gray squares) PMA are plotted against the holding potential (E_m). The PMA-induced reduction in I_{ss}/I_p was reduced at depolarized potentials.

effect on I_{ss} evoked by applying relatively intermediate concentrations of agonist and coagonist (50 μM NMDA and 0.5 μM glycine; Fig. 5A). In contrast, in the absence of EGTA PMA induced a clear depression of I_{ss} (Fig. 5B). Consequently, PMA caused a larger reduction in I_{ss}/I_p in recordings without EGTA than in those with EGTA (Fig. 5C), indicating that buffering the influx of Ca^{2+} reduced the degree of depression of I_{ss} . We then examined whether or not a Ca^{2+} buffer would block the PMA-induced depression of I_{ss} recorded in the presence of a near-saturating concentration of agonist and coagonist (300 μM NMDA and 3 μM glycine). To achieve this we used the more rapid Ca^{2+} buffer BAPTA (20 mM) in the patch pipette. As shown in Figure 5D the PMA-induced depression of I_{ss}/I_p was substantially reduced by BAPTA (two-way ANOVA, $p < 0.002$; Fig. 5D).

We also examined the relationship between I_{ss} and I_p under conditions of altered Ca^{2+} driving force to confirm the requirement of an influx of Ca^{2+} through NMDA channels. Before applying PMA (the intracellular solution contained 11 mM EGTA), the values of I_{ss}/I_p for the NMDA-evoked current were approximately the same at all holding potentials demonstrating that little Ca^{2+} inactivation was present. After treatment of the cells with PMA, the proportionate increase in I_p was the same at all potentials, but there was a disproportionate decrease in I_{ss} at negative holding potentials (Fig. 5E), conditions that favor a greater influx of Ca^{2+} via the channels. These results further support the interpretation that the PMA-induced reduction of I_{ss}/I_p is caused by enhancement of Ca^{2+} inactivation.

CaM depresses I_{ss} and occludes the effects of PMA

The binding of Ca^{2+} /CaM to the NR1 subunit of the NMDA receptor is thought to underlie Ca^{2+} inactivation of NMDA receptors (Zhang et al., 1998). Therefore, we added CaM (50–100 nM) to the patch pipettes and reexamined the effects of PMA in the presence of 0.2, 1.3, or 5.0 mM $[\text{Ca}^{2+}]_e$ (Fig. 6A). Addition of CaM caused a reduction of I_{ss}/I_p when compared with appropriate control responses.

Furthermore, the slope of the I_{ss}/I_p – $[\text{Ca}^{2+}]_e$ relationship was increased from -0.012 mM^{-1} (without CaM) to -0.031 mM^{-1} (Fig. 6B), demonstrating an enhanced sensitivity of the inactivation to Ca^{2+} . The subsequent addition of PMA further depressed I_{ss}/I_p but occluded the ability of PMA to enhance the Ca^{2+} sensitivity of inactivation (Fig. 6D,E).

We then examined the potential role of endogenous CaM in the PMA-induced depression of I_{ss} . A series of recordings was made using patch pipettes containing the inhibitory CaM-binding peptide KY9 or its ineffective control peptide KY8 (Liu et al., 1994). In the absence of PMA no changes in I_{ss}/I_p were observed over 30 min of recording regardless of whether KY9 or KY8 was included in the patch pipettes (Fig. 7A). The ratio of I_{ss} to I_p did decrease by $\sim 11\%$ (Fig. 7B) consistent with the time-dependent change in desensitization observed in control recordings (see Fig. 1B). In contrast, when PMA was applied the depression of I_{ss} was blocked in the presence of KY9 (200–300 μM), and PMA reduced I_{ss}/I_p by only 18% (before PMA, $I_{ss}/I_p = 0.5 \pm 0.03$; after PMA, $I_{ss}/I_p = 0.41 \pm 0.03$; Fig. 7C,E). In recordings with KY8 (300 μM) this ratio was reduced by $\sim 42\%$ (before PMA, $I_{ss}/I_p = 0.48 \pm 0.05$; after PMA, $I_{ss}/I_p = 0.28 \pm 0.04$; Fig. 7D,F).

We also included either the phosphatase inhibitor cyclosporin A (Tong et al., 1995) or the Ca^{2+} /CaM-dependent kinase II (CamK-II) inhibitor KN-93 in pipettes to determine whether the activity of these Ca^{2+} -dependent enzymes was required. The presence of cyclosporin A or KN-93 failed to prevent the PMA-induced Ca^{2+} -dependent increase in the sensitivity of inactivation (data not shown).

DISCUSSION

Phorbol esters such as PMA induce a complex regulation of NMDA receptors, acting to enhance I_p and simultaneously depressing I_{ss} evoked by applications of agonist. Both actions of PMA are mediated via activation of endogenous PKC because neither the enhancement nor the depression was mimicked by an

Figure 6. Intracellular applications of CaM suppressed I_{ss} and occluded the PMA-induced and Ca^{2+} -dependent reduction of I_{ss}/I_p . **A**, Example traces of original (top) and normalized (to peak; bottom) NMDA currents in the presence of three different $[\text{Ca}^{2+}]_e$. Currents before (thick lines) and after (thin lines) PMA are shown with CaM (50 nM) in the patch pipettes. **B**, Ratios of peak-to-steady-state currents recorded with the inclusion of CaM (gray circles; $n = 6$) are compared with similar recordings without CaM (Control, black circles; the same data used in Fig. 4D) and are plotted against $[\text{Ca}^{2+}]_e$. CaM reduced I_{ss}/I_p in a $[\text{Ca}^{2+}]_e$ -dependent manner (0.2 mM $[\text{Ca}^{2+}]_e$, Control, 0.55 ± 0.05 ; CaM, 0.42 ± 0.06 ; 1.3 mM $[\text{Ca}^{2+}]_e$, Control, 0.53 ± 0.02 ; CaM, 0.38 ± 0.05 ; $p < 0.05$; 5.0 mM $[\text{Ca}^{2+}]_e$, Control, 0.49 ± 0.08 ; CaM, 0.27 ± 0.06 ; $p < 0.05$). CaM enhanced the slope of the I_{ss}/I_p - $[\text{Ca}^{2+}]_e$ relationship from -0.012 mM^{-1} in control (thin line) to -0.031 mM^{-1} in CaM (thick line). **C**, PMA enhanced I_p in the presence (+CaM, black bars; $n = 6$) or absence (-CaM, white bars; the same data used in Fig. 4B) of CaM, and the degree of potentiation of I_p was similar at each $[\text{Ca}^{2+}]_e$. **D**, In contrast, intracellular CaM (black bars; $n = 6$) enhanced the PMA-induced depression of I_{ss} in the presence of low $[\text{Ca}^{2+}]_e$, while causing no further depression in the presence of high $[\text{Ca}^{2+}]_e$. **E**, With CaM in the patch pipettes, I_{ss}/I_p recorded before (CaM, gray circles) and after (CaM + PMA, black squares) PMA treatment is plotted against $[\text{Ca}^{2+}]_e$. Note that in the presence of CaM, PMA induced only a parallel shift of the I_{ss}/I_p - $[\text{Ca}^{2+}]_e$ relationship ($-PMA$, 0.031 mM^{-1} $[\text{Ca}^{2+}]_e$; $+PMA$, 0.03 mM^{-1} $[\text{Ca}^{2+}]_e$; $n = 6$), demonstrating that CaM occludes the Ca^{2+} -dependent inactivation of these currents.

inactive phorbol and both actions were blocked by chelerythrine. Previous experiments demonstrated that the potentiation of I_p depends on activation of Src (Lu et al., 1999) and $\text{CAK}\beta/\text{Pyk2}$ (Huang et al., 1999). In the present study we have confirmed that intracellular Src enhances I_p but also show that it fails to depress I_{ss} . Furthermore, the potentiation of I_p was blocked by the Src peptide Src(40–58), whereas the phorbol ester-induced depression of I_{ss} was not. Nor was the phorbol-induced depression of I_{ss} replicated by intracellular applications of the focal adhesion kinase $\text{CAK}\beta/\text{Pyk2}$. These results clearly demonstrate that the depression of I_{ss} depends on activation of endogenous PKC by a signaling cascade that bypasses the requirement for activation of Src and $\text{CAK}\beta/\text{Pyk2}$.

Glycine-independent desensitization

The major form of desensitization or inactivation of NMDA receptors in isolated CA1 neurons is a glycine- and calcium-independent form of desensitization (Sather et al., 1992; McBain and Mayer, 1994; Lu et al., 1998). This desensitization is accentuated as agonist or coagonist concentrations are increased (see Fig. 2), suggesting a concentration-dependent rate of entry into the desensitized state(s) of the receptor (Sather et al., 1992). In contrast, a glycine-sensitive component of desensitization predominates in whole-cell recordings from cultured cortical and hippocampal neurons (Mayer et al., 1989). In prolonged whole-cell recordings from cultured hippocampal neurons, the glycine-sensitive component of desensitization of NMDA-evoked currents is gradually lost (McBain and Mayer, 1994), revealing the glycine- and Ca^{2+} -insensitive component of desensitization (Sather et al., 1992; McBain and Mayer, 1994). A similar loss of this component is observed after excision of outside-out or nucleated patches (Sather et al., 1992; Tong and Jahr, 1994).

Generally, ratios of I_{ss}/I_p were decreased by PMA under all

conditions for which I_p and I_{ss} could be distinguished, implying a potential underlying alteration of glycine-independent desensitization. Furthermore, similar reductions in I_{ss}/I_p were observed in cultured neurons after the intracellular perfusion of a constitutively active PKC fragment (Xiong et al., 1998; Lu et al., 1999). Therefore, the depression of I_{ss} by phorbol ester might be accounted for on the basis of an enhancement of glycine-insensitive desensitization. However, our experiments strongly argue that this can be only a part of the explanation. For example, PMA depressed I_{ss} even when the potentiation of I_p was blocked by a Src inhibitor, and Src itself enhanced I_p without depressing I_{ss} . The depressant effects of PMA were also dependent on extracellular and intracellular concentrations of Ca^{2+} even though it is generally assumed that glycine-independent desensitization is Ca^{2+} independent (Sather et al., 1992; McBain and Mayer, 1994).

Unlike the glycine-independent form of desensitization, the loss of the glycine-dependent component of desensitization depends on intracellular Ca^{2+} and the activity of Ca^{2+} -dependent phosphatase (PP2B or calcineurin) (Tong and Jahr, 1994). Previously it was shown that the entry of Ca^{2+} through NMDA receptors acts via calcineurin to downregulate single-channel activity in cell-attached patch recordings from acutely isolated dentate gyrus neurons (Lieberman and Mody, 1994). An elevation of intracellular Ca^{2+} also accelerated the appearance of glycine-independent desensitization, and inhibitors of calcineurin retarded glycine-independent desensitization in cultured hippocampal neurons (Tong and Jahr, 1994). In contrast, neither activated PKC nor autophosphorylated CamK-II was able to regulate glycine-independent desensitization (Tong and Jahr, 1994). Calcium-induced activation of calcineurin together with a postulated dephosphorylation of NMDA receptors was suggested as the mechanism underlying synaptic desensitization of NMDA

Figure 7. The inhibitory CaM-binding protein KY9, but not the control peptide KY8, blocked the PMA-induced depression of I_{ss} . *A*, Example traces of NMDA-evoked currents recorded from the same cell 5, 15, and 25 min after establishment of the whole-cell patch configuration with KY9 in the pipette. *B*, Recordings from two groups of cells, one with KY9 (black circles; $n = 4$) and one with KY8 (gray squares; $n = 3$). I_{ss}/I_p was plotted against the time course of the recordings. *C*, NMDA currents recorded from the same cell shown in *A* (KY9). *Left*, Current recorded just before application of PMA (32 min after establishment of the whole-cell configuration). *Middle*, Current recorded after PMA treatment for 8 min. *Right*, The two traces superimposed. Note the blockade by KY9 of the PMA-induced depression of I_{ss} . *D*, NMDA-evoked currents recorded from another cell with KY8 in the patch pipette. *Left*, Current before application and recorded 36 min after establishment of the whole-cell configuration. *Middle*, Current recorded after PMA treatment for 6 min. *Right*, The two traces superimposed. Note the depression of I_{ss} by PMA. *E*, Summary of a series of recordings of I_{ss}/I_p with KY9 in the pipette before (control) and after PMA (control, 0.48 ± 0.03 ; PMA, 0.41 ± 0.03 ; $n = 6$). *F*, In another group of cells, I_{ss}/I_p recorded with KY8 (control, 0.45 ± 0.05 ; PMA, 0.28 ± 0.04 ; $p < 0.05$; $n = 5$).

receptors (Tong et al., 1995), and rephosphorylation of the receptor by PKA was suggested as the mechanism of its recovery (Raman et al., 1996).

PKC enhances Ca^{2+} -dependent inactivation of NMDA-evoked currents

Changing $[\text{Ca}^{2+}]_e$ from 0.2 to 5 mM only slightly reduced I_{ss}/I_p (Fig. 4C), demonstrating that with 11 mM EGTA in the patch pipette hippocampal CA1 neurons exhibit little Ca^{2+} -dependent inactivation (Lu et al., 1998). Nonetheless, the presence of a Ca^{2+} -dependent inactivation (an decrease in I_{ss}/I_p) of these currents was revealed after applications of PMA. The proportionate enhancement of I_p by PMA was approximately the same in all concentrations of extracellular Ca^{2+} , showing that the depression of I_{ss} and I_{ss}/I_p after activation of PKC was not likely a consequence of an enhanced glycine-independent desensitization. This result is also consistent with our previous demonstration that constitutively active PKC enhances I_p regardless of the presence or absence of $[\text{Ca}^{2+}]_e$ (Xiong et al., 1998). Elevating extracellular Ca^{2+} or membrane hyperpolarization, both of which favor entry of Ca^{2+} through NMDA channels, increased the depression of I_{ss} , whereas increased intracellular buffering of Ca^{2+} reduced it. Therefore, the most parsimonious explanation for our observations is that PMA acting via endogenous PKC enhances the sensitivity of Ca^{2+} -dependent inactivation of NMDA receptor-mediated currents.

Ca^{2+} inactivation of NMDA channels results from an inhibition of channel gating associated with the binding of $\text{Ca}^{2+}/\text{CaM}$ to the carboxy tail of the NR1 subunit (Zhang et al., 1998; Krupp et al., 1999). In agreement with this mechanism we found that intracellular application of CaM to isolated CA1 neurons reduced I_{ss}/I_p , indicative of an enhanced Ca^{2+} -dependent inactivation of these currents. Also the residual PMA-induced inhibition observed in the presence of CaM proved to be independent of $[\text{Ca}^{2+}]_e$, supporting the interpretation that CaM occludes PMA-induced Ca^{2+} -dependent inactivation. This evidence also dem-

onstrates that a part of the effect of PMA is mediated via a Ca^{2+} -independent inhibition of NMDA-evoked currents.

Our demonstration that the inhibitory CaM-binding peptide KY9 blocked the PMA-induced depression of I_{ss} provides strong support for the role of endogenous CaM in the PKC-induced potentiation of Ca^{2+} inactivation. Previously, inclusion of KY9 in patch pipettes was reported to depress I_p recorded from human embryonic kidney 293 cells transfected with cDNAs for NMDA channel subunits (Zhang et al., 1998). This result was interpreted as a blockade of Ca^{2+} inactivation because the reduction of I_p resulted in an increase of I_{ss}/I_p . Krupp et al. (1999) disputed this interpretation and considered the depression of I_p to be a non-specific action resulting from the putative amphipathic helical structure of CaM inhibitory peptides. However, using intracellular applications of KY9, we have demonstrated that this peptide was itself without effect on either the peak or steady-state currents evoked by applications of NMDA to CA1 neurons, whereas it was effective at blocking the PMA-induced enhancement of Ca^{2+} inactivation.

The NR1a subunit possesses two binding sites for CaM, a high-affinity site in the C1 cassette and a low-affinity site in the C0 region (Ehlers et al., 1996b); however, it is the low-affinity site that is responsible for inhibition of channel gating. Inactivation likely results in part from displacement of the cytoskeletal-linking protein α -actinin by $\text{Ca}^{2+}/\text{CaM}$ bound to the same C0 site (Krupp et al., 1999). The function of CaM binding to the high-affinity site is unknown. In hippocampal neurons it was shown recently that PKC phosphorylates residues in the C1 cassette of the NR1 subunit and reduces but does not eliminate CaM binding to the NR1 subunit (Hisatsune et al., 1997). These results appear to be contrary to our observations that PKC enhances $\text{Ca}^{2+}/\text{CaM}$ -dependent inactivation.

To interpret this contradiction, it should be noted that the residues of PKC-induced phosphorylation are in the C1 and not the C0 cassette where binding of CaM is associated with inhibition of channel gating. One possible explanation is that phosphor-

ylation in the C1 cassette displaces CaM at this site, providing a source of CaM for binding at the C0 site and leading to an enhancement of channel inactivation. In this way the NR1 subunit might function as a CaM-binding protein, mimicking proteins such as neurogranin (RC3), neuromodulin (GAP-43), and the myristoylated; alanine-rich C kinase substrate (Chakravarthy et al., 1999). The binding of CaM or Ca^{2+} /CaM to these proteins is also inhibited by PKC phosphorylation. Alternatively, PKC-induced phosphorylation within the C1 cassette of the NR1 may enhance the sensitivity of the C0 cassette to the binding of Ca^{2+} /CaM or reduce the binding of α -actinin.

Moreover, effects of PKC on NMDA-evoked currents can be affected by interactions between the channel and postsynaptic density proteins or cytoskeletal elements. For example, coexpression of NMDA receptor subunits with PSD-95 in oocytes greatly reduces the degree of enhancement by phorbol esters of NMDA-evoked currents (Yamada et al., 1999). Furthermore, in neurons both CaM and activation of PKC inhibit the binding of the cytoskeleton-linking protein spectrin to the C terminal of the NR1 subunit (Wechsler and Teichberg, 1998). The PKC-dependent phosphorylation of the NR1 subunit also inhibits clustering subunits overexpressed in a cell line (Ehlers et al., 1995; Tingley et al., 1997). Therefore, PKC-induced phosphorylation of NR1 subunits may enhance dissociation of NMDA channels from their linking cytoskeletal proteins, subsequently affecting channel location and physical proximity to other Ca^{2+} -dependent signaling enzymes (Sattler et al., 1999). Alternatively, PKC may phosphorylate CaM, α -actinin, or other cytoskeletal proteins than in turn modulate the sensitivity of Ca^{2+} -dependent inactivation of NMDA receptors.

The role of Ca^{2+} inactivation in determining the time course of synaptic events is not well understood. However, an influx of Ca^{2+} induced by repetitive firing can depress the NMDA receptor-mediated component of synaptic transmission between individual cultured neurons (Medina et al., 1999). Desensitization of NMDA receptors may also contribute directly to the time course of synaptic responses (Huganir and Greengard, 1990; Jones and Westbrook, 1995; Paradiso and Brehm, 1998). Phosphorylation of NMDA receptors may regulate both their desensitization (Tong and Jahr, 1994; Tong et al., 1995) and their inactivation by an influx of Ca^{2+} , providing multiple mechanisms to limit the time course of excitatory synaptic currents.

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