Phorbol 12-Myristate 13-Acetate Potentiation of *N*-Methyl-D-aspartate-Induced Currents in Primary Cultured Cerebellar Granule Cells Is Mediated by Protein Kinase C α

Jason C. Reneau, Mary E. Reyland, Jonathan Phillips, Carissa Kindy, and R. Lisa Popp

Department of Pharmacology and Neuroscience (J.C.R., J.P., C.K., R.L.P.), South Plains Alcohol and Addiction Research Center (R.L.P.), Texas Tech University Health Sciences Center, Lubbock, Texas; and Department of Craniofacial Biology, University of Colorado Denver, Aurora, Colorado (M.E.R.)

Received March 5, 2009; accepted May 7, 2009

ABSTRACT

We have previously reported that activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) results in potentiation of *N*-methyl-D-aspartate-induced currents (I_{NMDA}) of receptors contained in primary cultured cerebellar granule cells (CGCs). The purpose of this study was to identify which PKC isoform(s) was responsible for this effect by using the whole-cell patch-clamp technique. Experiments were conducted on CGCs that expressed both the NR2A and NR2B NMDA receptor subunits as well as the PMA-sensitive PKC isoforms α , β I, β II, δ , ϵ , γ , and θ . As observed previously, *N*-methyl-D-aspartate-induced peak currents (I_{PK}) were enhanced by a 12.5-min, 100 nM PMA exposure at 37°C under normal recording conditions. Potentiation of receptor function was not observed when extracellular Ca^{2+} was removed and 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid was present inside the

cell. PMA-induced potentiation of I_{PK} did not occur when PKC α -specific antibody was introduced into the cell via the recording electrode. However, in similar experiments with antibodies specific for PKC β II, δ , ϵ , γ , and θ , PMA potentiation of I_{PK} was observed. Down-regulation of PMA-sensitive PKC isoforms by an overnight exposure of 100 nM PMA resulted in lack of potentiation by PMA that was rescued when catalytically active PKC α was introduced into the cell via the patch electrode. PMA potentiation of I_{PK} was not recovered when catalytically active PKC β I, PKC β II, or PKC γ was introduced into the cell via the patch electrode. Collectively, our data provide strong evidence that PMA-enhanced function of *native* NMDA receptors expressed in primary cultured CGCs is mediated by activation of PKC α .

N-Methyl-D-aspartate (NMDA) receptors are heteromeric proteins activated by glutamate and the coagonist glycine (Kleckner and Dingledine, 1988). Native receptors are composed of proteins from three gene families (Moriyoshi et al., 1991; Monyer et al., 1994; Ciabarra et al., 1995; Chatterton et al., 2002): NR1 of which there are eight splice variants of one gene, NR2 A to D, and NR3 A and B. It is well documented that protein kinase C (PKC), a family of serine-threonine kinases, phosphorylates NR1 (Tingley et al., 1993, 1997) as well as the NR2A and NR2B subunits of this receptor (Leonard and Hell, 1997). Furthermore, activation of PKC by phorbol esters or by constitutively active PKC results in

enhancement of NMDA receptor function. Augmentation of NMDA receptor function by activation of PKC has been observed in both oocytes (Yamakura et al., 1993; Sigel et al., 1994; Zheng et al., 1997, 1999; Lan et al., 2001; Liao et al., 2001) and human embryonic kidney cells (Grant et al., 1998; Xiong et al., 1998) transiently expressing NMDA receptors. Augmentation of native NMDA receptors has been reported in hippocampal neurons (Xiong et al., 1998; Lu et al., 1999; Lan et al., 2001); in spinal cord and brain stem neurons (Gerber et al., 1989; Chen and Huang, 1991, 1992); and by our laboratory, in primary cultured cerebellar granule cells (CGCs) (Popp et al., 2008a). We observed that phorbol 12-myristate 13-acetate (PMA) augmented NMDA-induced peak current (I_{PK}) amplitude as well as NMDA-induced steady-state current (I_{SS}) amplitude of receptors expressed in 13 to 15 days in vitro (DIV) CGCs. PMA treatment did not affect the desensitized state of the receptor as indicated by no

This work was supported by the National Institutes of Health [Grants R01-AA13436, 2R01-DE015648] (to R.L.P. and M.E.R., respectively).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.109.153163.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; CGC, cerebellar granule cell; PMA, phorbol 12-myristate 13-acetate; I_{PK} , *N*-methyl-D-aspartate-induced peak current(s); I_{SS} , *N*-methyl-D-aspartate-induced steady-state current(s); $I_{\text{PK}}/I_{\text{SS}}$, steady-state to peak current ratio(s); DIV, days in vitro; DAG, diacylglycerol; ON, overnight; DMSO, dimethyl sulfoxide; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; ANOVA, analysis of variance; nACh, nicotinic acetylcholine.

change in steady-state to peak current ratios (I_{PK}/I_{SS}) (Popp et al., 2008a). Yet, the identification of the PKC isoform or isoforms responsible for NMDA receptor augmentation was not identified in these studies.

We have confirmed previously that the NR2A and NR2B NMDA receptor subunit proteins are present in our primary cultured CGCs between 10 and 15 DIV (Popp et al., 1999, 2008b). This combination of NMDA receptor subunits is observed in the neonatal rat cerebellum (Farrant et al., 1994; Monyer et al., 1994). We have further confirmed through the use of PKC isoform-specific antibodies that nine of the 10 isoforms of this phospholipid-dependent kinase are expressed in CGCs during this time in vitro (Popp et al., 2006). PKC isoforms have been grouped according to sequence homology, substrate preference, and activators (Newton, 2001; Ohno and Nishizuka, 2002). Expressed in our CGCs from 13 to 15 DIV are the classical PKC isoforms α , β I, β II, and γ that require calcium for activation, and activation is enhanced by diacylglycerol (DAG) and phorbol esters and the novel PKC isoforms δ , ϵ , and θ that are also enhanced by DAG and phorbol esters but do not require calcium for activation. The atypical PKC isoforms ζ and λ are also expressed in our CGCs during this time in culture, but because activation of these isoforms is not influenced by calcium, DAG, or phorbol esters, they could not be responsible for PMA augmentation of NMDA receptor function.

The purpose of this study was to identify with the whole-cell patch-clamp method which PKC isoform or isoforms are responsible for PMA-induced augmentation of NMDA receptor function of receptors expressed in primary cultured CGCs. With PMA treatment conditions (Popp et al., 2008a) that previously resulted in a robust time-dependent enhancement of NMDA-induced currents, we were able to identify experimental conditions that abrogated PMA enhancement of I_{PK} . Furthermore, our results suggest that PKC α is the sole isoform responsible for PMA enhancement of I_{PK} of NMDA receptors expressed in primary cultured CGCs.

Materials and Methods

Preparation and Maintenance of CGC Cultures. All culture procedures were carried out in accordance with guidelines contained in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (NIH Publication 8023, revised 1978). Culturing of CGCs has been described in detail previously (Popp et al., 1999). In brief, all drugs were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Primary neuronal cultures were prepared using cerebellar tissue from 5- to 9-day-old Sprague-Dawley rats (Charles River, Raleigh, NC). The tissue was enzymatically digested, and the cells (500,000–550,000 cells/ml) were dissociated by trituration and plated onto poly-D-lysine (5 μ g/ml)-coated 35-mm dishes in plating medium. The plating medium consisted of minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine, 130 μ M DNase, 10% heat-inactivated horse serum, and 10% fetal bovine serum. After 18 to 20 h, this medium was replaced with feeding medium [minimal essential medium containing 5% fetal bovine serum, 2 mM L-glutamine, 25 mM KCl (to promote CGC viability in culture; Gallo et al., 1987), penicillin/streptomycin (100 U/100 μ g/ml), and a fluorodeoxyuridine/uridine (35 μ M/15 μ M) mixture]. To minimize excitotoxic cell death due to the introduction of fresh medium, we did not exchange the medium, but the existing medium was supplemented with 0.5 ml of fresh feeding medium every 7 days. This method of CGCs maintenance in vitro results in very little cell death as indicated by the trypan blue

exclusion method (Popp et al., 1999) and results in CGCs that are smooth, almond-shaped neurons with many neuritic process and are phase bright (Popp et al., 2008b) for up to 28 to 35 DIV. For the experiments presented, cells were maintained at 37°C in 5% CO₂ for up to 15 DIV.

Kinase Activity Assays. Each kinase (50 ng) was diluted in 20 μ l of 2 $\times$ kinase buffer (40 mM Tris, pH 7.4, 20 mM MgCl₂, 20 μ M nonradioactive ATP, and 2.5 mM CaCl₂). To begin the reaction, 20 μ l of a kinase reaction cocktail [50 μ g/ml phosphatidylserine, 4 μ M dileoylglycerol, 5 μ Ci of [γ -³²P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA), 0.5 mg/ml histone H1 (Sigma Chemical Co.)] was added, and the reaction was incubated for 10 min at 30°C. Reactions were terminated by the addition of 2 $\times$ Laemmli sample buffer, samples were heated to >90°C for 4 min, and the reaction products were resolved on a 12.5% SDS-polyacrylamide gel. The extent of histone H1 phosphorylation was determined by autoradiography and quantified using a PhosphorImager (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The data are expressed as relative units (acquired from the PhosphorImager) per 50 ng of purified PKC (for PKC α , 3.9 and 50 ng were assayed). Baculovirus-expressed and purified His-tagged PKC δ was included as a positive control for the assay.

Drug Treatment for Electrophysiological Recordings. At 13 to 15 DIV, each dish of CGCs was treated with 100 nM PMA for either 12.5 min or overnight (ON). All drug treatments were conducted at 37°C. Stock PMA in dimethyl sulfoxide (DMSO) solutions was diluted to 100 nM (0.001% DMSO) and applied in conditioned cell media. We have reported previously that this concentration of DMSO does not result in translocation of PKC immunoreactivity for up to 18 to 20 h of exposure (overnight exposure) (Popp et al., 2006). NMDA-induced currents from receptors contained in 13 to 15 DIV CGCs when exposed to this concentration of DMSO (overnight) do not differ from NMDA-induced currents from control cells and are enhanced when challenged with 30-min, 100 nM PMA treatment at 37°C the next day (Popp et al., 2008a), indicating that increases in I_{NMDA} are due to PMA and not the vehicle DMSO. Dishes receiving an overnight PMA treatment were treated and returned to the incubator until the next day at which time they received a second 100 nM PMA dose for 30 min (ON PMA challenge). This treatment results in the down-regulation of PMA-sensitive PKC isoforms (Mathies et al., 1987; Popp et al., 2006, 2008a). Alternatively, dishes not receiving an overnight PMA treatment were treated once with 100 nM PMA and returned to the incubator for 12.5 min. All experiments were conducted from a minimum of three different culture batches, and data were acquired from one cell/dish. After PMA treatment, dishes were washed for 30 to 45 min in either normal or Ca²⁺-free external recording solution at 23°C; normal recording solution consists of 150 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 10 mM HEPES, 10 mM D-glucose, and 200 nM tetrodotoxin (Alomone Labs, Jerusalem, Israel), with pH adjusted to 7.4 with NaOH and osmolality adjusted to 333 to 345 mOsmol with sucrose. Cells were patched with normal patch electrode solution containing 100 mM N-methyl-D-glucamine, 100 mM MeSO₃, 40 mM cesium fluoride, 10 mM HEPES, 1 mM MgCl₂, 3 mM lidocaine N-ethyl bromide, and 5 mM EGTA, with pH adjusted to 7.4 with CsOH and osmolality adjusted to 314 to 317 mOsmol with sucrose. In experiments to access whether Ca²⁺ was required for PMA-induced augmentation of I_{NMDA} , 2.5 mM BaCl₂ replaced CaCl₂ (calcium-free external recording solution) and 10 mM BAPTA (Tocris Bioscience, Ellisville, MO) replaced EGTA in the patch electrode solution.

To determine which PKC isoform(s) was responsible for PMA-induced changes in I_{NMDA} , cells were patched with one of the following antibodies diluted in normal (no BAPTA) internal patch electrode solution: monoclonal anti-PKC α , 1:1000; polyclonal anti-PKC β II, 1:1000; polyclonal anti-PKC γ , 1:1000; polyclonal anti-PKC δ , 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); monoclonal anti-PKC ϵ , 1:2500; and monoclonal anti-PKC θ , 1:1000 (BD Biosciences Transduction Laboratories, Lexington, KY). CGCs in which PMA-

sensitive PKC isoforms had been down-regulated (ON PMA challenge) were conducted in normal external recording solution and patched with either normal patch electrode solution or normal patch electrode solution containing 1 $\mu\text{g/ml}$ catalytically active PKC α or PKC β or PKC δ or PKC γ (Millipore, Billerica, MA). Diluted amounts of PKC α to compensate for a higher specific activity compared with the other isoforms were 0.1 and 0.05 $\mu\text{g/ml}$ and were also diluted in normal patch electrode solution.

Whole-Cell Patch-Clamp Recordings. Dishes were placed on an Eclipse TE2000-U inverted microscope (Nikon Inc., Melville, NY). All recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) at -60 mV holding potential. Detailed methods have been published previously (Popp et al., 1999). In brief, dishes were superfused at 1 to 2 ml/min throughout recording with normal or Ca^{2+} -free external recording solution. Agonists (100 μM NMDA and 10 μM glycine) were dissolved in normal or Ca^{2+} -free external solution and delivered to the patched cell by gravity through microcapillary tubes (0.32-mm inner diameter) operated manually. With this drug application system, our solution exchange rate is faster than the time to reach peak current amplitudes and thus can be used to measure changes in I_{PK} (Popp and Dertien, 2008). We had reported previously that 100 nM PMA exposure at 37°C resulted in augmentation of I_{NMDA} (both I_{PK} and I_{SS}) and that significant increases in I_{PK} , but not I_{SS} were time-dependent (Popp et al., 2008a). Therefore, we used this time-dependent effect of PMA on I_{PK} enhancement to identify the PKC isoform(s) mediating this effect. NMDA-induced current traces (three to four) were derived from agonists application every 30 s for a period of 5 s. In an attempt to negate the confound of glycine-insensitive desensitization due to repetitive agonist exposure, these clusters of agonist application were followed by a 2-min break during which time the patched cell was bathed with external recording solution. This agonist application paradigm was repeated up to 20 min. Data were low pass-filtered at 1 kHz with an eight-pole Bessel filter (Axopatch 200B amplifier) at a sampling rate of 10 kHz with a DigiData 1200A and Clampex 9.2 (Molecular Devices). Only data sets containing current traces with a steady baseline and constant series resistance throughout the experiment were used for analysis.

Statistical Analysis. All data are expressed as mean $\pm$ S.E.M. and were acquired with Clampfit 9.2 software (Molecular Devices). The time-dependent effect of PMA on I_{PK} and I_{SS} was assessed by obtaining the mean value from currents obtained from agonist clusters at time points 0, 4, 8, 12, 16, and 20 min, with 0 being the initial set of agonist-induced currents. Values were normalized to cell capacitance (pA/pF). A one-way analysis of variance (ANOVA) compared differences among the time points for I_{SS} , I_{PK} , or $I_{\text{SS}}/I_{\text{PK}}$, but data for only time points 0, 4, 12, and 20 min are reported. Pairwise comparisons when significant differences (F values resulted in $P \leq 0.05$) were reported were determined by the multiple t test (Bartlett, 1976).

Results

PMA-Induced Potentiation of NMDA Receptor Function Is Calcium-Dependent. We have reported previously that a 100 nM PMA exposure at 37°C results in increases in both NMDA-induced steady-state and peak current amplitudes (I_{NMDA}) for receptors expressed in primary cultured CGCs 13 to 15 DIV (Popp et al., 2008a). These increases are time-dependent for I_{PK} but not for I_{SS} in that both a longer PMA exposure time as well as a sustained increase in I_{PK} over recording time are observed, whereas increases in I_{SS} plateau after a 12.5-min PMA exposure and seemed not to significantly increase over recording time. To begin to identify which PKC isoform(s) is responsible for this effect, we expanded the above-mentioned observation in that we con-

tinually monitored PMA-induced changes in I_{NMDA} amplitudes for up to 20 min during whole-cell patch-clamp recordings with different experimental paradigms. Clusters of I_{NMDA} were obtained every 2 min with normal recording solution and normal patch electrode solution. Over the course of the 20-min recording, I_{PK} amplitudes significantly increased [$F_{(5,48)} = 14.8$; $P \leq 0.001$] (Fig. 1A). Post hoc analysis indicated PMA significantly increased I_{PK} from 57.64 ± 0.9 pA/pF at 0 min to 65.19 ± 1.9 pA/pF at 4 min ($t = 3.4$, $P \leq 0.01$), 69.32 ± 1.0 pA/pF at 12 min ($t = 5.3$, $P \leq 0.001$), and 72.48 ± 0.8 pA/pF at 20 min ($t = 6.7$, $P \leq 0.001$) (Fig. 1B). Potentiation of I_{PK} plateaued at 8 min because this is the last time point at which increases in I_{PK} significantly differed from the 20-min time point ($t = 3.4$, $P \leq 0.01$). However, unlike our previous observations (Popp et al., 2008a) I_{SS} did not significantly increase with this agonist exposure paradigm after PMA treatment [$F_{(5,48)} = 0.1$; $P = 0.98$]. Although not significant, modest increases in I_{SS} prevented changes in receptor desensitization as indicated by nonsignificant differences in $I_{\text{SS}}/I_{\text{PK}}$ among the time points [$F_{(5,48)} = 0.49$; $P = 0.77$]. This lack of PMA-induced increase in NMDA receptor desensitization is identical to previously published results by our laboratory, and it would seem that the only difference from previous experiments is the lack of significantly enhanced I_{SS} due to PMA treatment (Popp et al., 2008a).

To determine whether PMA-induced changes in I_{PK} were mediated by Ca^{2+} -dependent PKC isoforms, experiments were repeated in Ca^{2+} -free external recording solution and internal patch electrode solution containing 10 mM BAPTA. This concentration of BAPTA has been shown to block potentiation of I_{NMDA} mediated by PKC activation (Tyszkiewicz et al., 2004). As shown in Fig. 1, C and D, PMA exposure did not enhance I_{PK} [$F_{(5,42)} = 0.13$; $P = 0.98$] at any time point up to

Fig. 1. PMA augmentation of I_{PK} is abolished under Ca^{2+} -free recording conditions. Clusters of NMDA-induced current traces were recorded between 2-min breaks and averaged at 0-, 4-, 12-, and 20-min time points. A, representative current traces taken from a 14 DIV CGC, indicating sustained augmentation of I_{PK} over time as a result of treatment with 12.5-min, 100 nM PMA (37°C). Data were taken from nine cells and are summarized in B (mean $\pm$ S.E.M.). Lack of CaCl_2 in the external recording solution and substitution of EGTA with BAPTA in the internal patch solution eliminated PMA-induced changes in I_{PK} . C, representative current traces taken from a 13 DIV CGC in the absence of calcium. Peak current amplitude does not change throughout the 20-min recording. Data from eight different cells are summarized in D (mean $\pm$ S.E.M.). **, $P \leq 0.01$; ***, $P \leq 0.001$.

20 min under these recording conditions. I_{SS} [$F_{(5,42)} = 0.42$; $P = 0.82$] and I_{SS}/I_{P_k} [$F_{(5,42)} = 0.54$; $P = 0.74$] were also not altered by PMA exposure under these recording conditions.

PMA Potentiation of I_{P_k} Is Not Mediated by the Novel PKC Isoforms δ , ϵ , and θ . Results from the previous experiments suggested that the activation of a Ca^{2+} -dependent (classical) PKC isoform might be responsible for PMA-induced potentiation of I_{P_k} . Antibodies directed against the $\alpha 7$ -nicotinic acetylcholine (nACh) receptor have been introduced into the cell during whole-cell patch-clamp experiments to block acetylcholine-evoked currents in superior cervical ganglion neurons (Cuevas et al., 2000). We used a similar technique to confirm that PMA potentiation of I_{P_k} was indeed mediated by a classical PKC isoform by introducing PKC isoform-specific antibodies (Popp et al., 2006) into the cell during whole-cell recordings. When antibodies directed against the novel, noncalcium-dependent PKC isoforms δ , ϵ , and θ were introduced into the cell via the patch electrode, time-dependent potentiation of I_{P_k} by PMA was still observed (Fig. 2). After a 12.5-min, 100 nM PMA (37°C) exposure, cells were patched and recorded as described under

Fig. 2. PMA-enhancement of I_{P_k} is unaffected by intracellular dialysis of antibodies directed against PKC δ , ϵ , and θ . Antibodies were added to the internal patch solution at concentrations specific for their respective antibody. A, representative current traces taken from a 15 DIV CGC in which potentiation of I_{P_k} due to 100 nM PMA treatment for 12.5 min at 37°C was significantly enhanced up to 20 min with anti-PKC δ (1:1000) present in the patch solution. Data were obtained from eight different cells and are summarized in B (mean $\pm$ S.E.M.). Adding anti-PKC ϵ (1:2500) also did not affect PMA-induced potentiation of I_{P_k} . C, representative current traces from a 13 DIV CGC showing a steady and significant increase in I_{P_k} up to 20 min. D, graph of summarized data from eight different cells (mean $\pm$ S.E.M.). With anti-PKC θ (1:1000) in the patch solution, potentiation of I_{P_k} continued to increase steadily. E, representative current traces obtained from a 13 DIV CGC showing an increase in I_{P_k} similar to that observed when anti-PKC δ and ϵ were present inside the cell during whole-cell recordings. Data from nine cells are summarized in F (mean $\pm$ S.E.M.). **, $P \leq 0.01$; ***, $P \leq 0.001$.

Materials and Methods with anti-PKC δ , anti-PKC ϵ , or anti-PKC θ in the patch solution. Potentiation of I_{P_k} was significantly increased over time despite intracellular dialysis with anti-PKC δ [$F_{(5,42)} = 3.7$; $P \leq 0.01$] (Fig. 2A). I_{P_k} increased from 54.39 ± 3.6 pA/pF at 0 min to 59.16 ± 3.4 pA/pF at 4 min ($t = 1.08$, $P \geq 0.05$), 67.07 ± 2.9 pA/pF at 12 min ($t = 2.8$, $P \leq 0.01$), and 70.16 ± 3.6 pA/pF at 20 min ($t = 3.5$, $P \leq 0.001$) (Fig. 2B). I_{P_k} plateaued at 4 min because this was the last significant time point at which I_{P_k} differed from the 20-min time point ($t = 2.5$, $P \leq 0.05$). Similar results were also observed when anti-PKC ϵ and anti-PKC θ were dialyzed into the cell during whole-cell recordings [$F_{(5,42)} = 7.6$; $P \leq 0.001$ and $F_{(5,42)} = 10.5$; $P \leq 0.001$ for anti-PKC ϵ and anti-PKC θ , respectively] (Fig. 2, C and E). In the presence of anti-PKC ϵ , PMA potentiation of I_{P_k} increased from 50.72 ± 3.5 pA/pF at 0 min to 55.43 ± 3.7 pA/pF at 4 min ($t = 1.0$, $P \geq 0.05$), 66.20 ± 3.6 pA/pF at 12 min ($t = 3.3$, $P \leq 0.01$), and 74.79 ± 2.9 pA/pF at 20 min ($t = 5.2$, $P \leq 0.001$). I_{P_k} plateaued at 8 min ($t = 2.4$, $P \leq 0.05$ between 8 and 20 min) (Fig. 2D). For cells that had been dialyzed with anti-PKC θ , PMA potentiated I_{P_k} from 47.77 ± 2.3 pA/pF at 0 min to 53.12 ± 1.9 pA/pF at 4 min ($t = 1.7$, $P \geq 0.05$), 60.88 ± 2.3 pA/pF at 12 min ($t = 4.1$, $P \leq 0.001$), and 67.53 ± 2.8 pA/pF at 20 min ($t = 6.3$, $P \leq 0.001$) (Fig. 2F). Increases in I_{P_k} plateaued at 12 min given that this was the last significant difference ($t = 2.1$, $P \leq 0.05$) in I_{P_k} from the 20-min time point. For all three novel PKC isoforms, under these recording conditions, I_{SS} did not significantly change; yet, modest increases in I_{SS} were observed when anti-PKC δ was present inside the cell, and this resulted in nonsignificant differences in I_{SS}/I_{P_k} . However, this was not the case when anti-PKC θ and anti-PKC ϵ were present inside the cell in that the increase in I_{P_k} with no change in I_{SS} resulted in a significant decrease in I_{SS}/I_{P_k} . During the 20-min recording, I_{SS}/I_{P_k} decreased from 0.57 to 0.48 and from 0.39 to 0.24 for PKC θ and PKC ϵ , respectively. Data are summarized in Table 1.

Intracellular Perfusion of Anti-PKC α but Not Perfusion with Anti-PKC β II or Anti-PKC γ Prevents PMA Augmentation of NMDA Receptor Function. To determine whether PMA-mediated potentiation of NMDA receptor function was mediated by one or more of the classical PKC isoforms, similar experiments were conducted with antibodies directed against PKC α , β II, or γ dialyzed into the cell via the patch electrode. As shown in Fig. 3, A and B, I_{P_k} increased steadily at each time point up to 20 min when anti-PKC β II was in the patch solution [$F_{(5,42)} = 10.0$; $P \leq 0.001$].

TABLE 1

Desensitization of NMDA receptors expressed in primary cultured CGCs may be mediated by specific novel PKC isoforms

Results from a one-way ANOVA indicating that a 12.5-min PMA exposure at 37°C does not produce a significant time-dependent increase in NMDA-induced I_{SS} between 0- and 20-min post-PMA exposure when antibodies directed against the novel PKC isoforms δ , ϵ , and θ are present intracellularly during whole-cell recordings. This result in combination with significant increases in NMDA-induced peak current amplitudes led to an increase in receptor desensitization as indicated by significant changes I_{SS}/I_{P_k} for anti-PKC ϵ and anti-PKC θ but not for anti-PKC δ .

	PKC Isoform	df	F Value	P Value
I_{SS}	δ	5,48	0.03	N.S.
I_{SS}/I_{P_k}	δ	5,48	2.2	N.S.
I_{SS}	ϵ	5,42	0.08	N.S.
I_{SS}/I_{P_k}	ϵ	5,42	3.2	0.05
I_{SS}	θ	5,42	1.7	N.S.
I_{SS}/I_{P_k}	θ	5,42	2.5	0.04

Fig. 3. PMA potentiation of I_{P_k} is blocked when antibodies directed against PKC α are dialyzed into the cell. CGCs were treated with PMA as described in Fig. 2 legend. Introduction of anti-PKC β II (1:1000) into the cell via the patch solution did not abrogate continued potentiation of I_{P_k} by PMA over the 20-min time frame. A, representative current trace taken from a 15 DIV CGC, and summarized data (mean $\pm$ S.E.M.) from eight cells are shown in B. Anti-PKC γ (1:1000) also had no effect on attenuating PMA-induced potentiation of I_{P_k} . C, representative current traces showing an increase in I_{P_k} at each consecutive time point taken from a 14 DIV CGC. D, summary graph showing data from nine cells (mean $\pm$ S.E.M.). The addition of anti-PKC α (1:1000) into the cell via the patch solution abrogated the time-dependent potentiation of I_{P_k} by PMA. E, representative current traces taken from a 13 DIV CGC. One-way ANOVA indicated no significant difference between time points. Data from nine different cells are summarized in F (mean $\pm$ S.E.M.). **, $P \leq 0.01$; ***, $P \leq 0.001$.

PMA significantly increased I_{P_k} from 50.65 ± 2.2 pA/pF at 0 min to 57.50 ± 1.8 pA/pF at 4 min ($t = 1.5$, $P \geq 0.05$), 69.09 ± 3.9 pA/pF at 12 min ($t = 4.1$, $P \leq 0.001$), and 77.70 ± 4.3 pA/pF at 20 min ($t = 6.0$, $P \leq 0.001$). Increases in I_{P_k} plateaued at 8 min ($t = 2.8$, $P \leq 0.01$ between I_{P_k} values for 8- and 20-min time points and no significant differences observed at later times). Similar results were observed when anti-PKC γ was present in the patch solution (Fig. 3, C and D). I_{P_k} potentiation increased significantly [$F_{(5,48)} = 4.2$; $P \leq 0.01$] from 0 to 20 min: 56.2 ± 1.6 pA/pF at 0 min to 62.99 ± 2.7 pA/pF at 4 min ($t = 1.6$, $P \geq 0.05$), 69.37 ± 3.4 pA/pF at 12 min ($t = 3.2$, $P \leq 0.01$), and 71.1 ± 3.6 pA/pF at 20 min ($t = 3.6$, $P \leq 0.001$). At the 8-min time point, I_{P_k} remained elevated but did not significantly increase ($t = 0.09$, $P \geq 0.05$ for I_{P_k} values at the 8- and 20-min time points). Similar to the results from experiments with the novel PKC isoforms, I_{SS} was not significantly enhanced under these recording conditions: $F_{(5,42)} = 0.22$; $P = 0.94$ and $F_{(5,48)} = 0.27$; $P = 0.9$ for PKC β II and PKC γ , respectively. Unlike the novel PKC isoforms, modest increases in I_{SS} resulted in no significant alterations in I_{SS}/I_{P_k} for PKC β II [$F_{(5,42)} = 1.51$; $P = 0.2$] and

PKC γ [$F_{(5,48)} = 0.79$; $P = 0.55$]. However, cells patched with anti-PKC α contained in the patch solution showed no significant increase in PMA-induced potentiation over the course of the 20-min recording for I_{P_k} [$F_{(5,48)} = 0.58$; $P = 0.7$], I_{SS} [$F_{(5,48)} = 0.8$; $P = 0.55$], and I_{SS}/I_{P_k} [$F_{(5,48)} = 1.8$; $P = 0.12$] (Fig. 3, E and F). PKC β I is a classical PKC isoform expressed in our CGCs during the first 2 weeks in culture (Popp et al., 2006). The method of intracellular dialysis with anti-PKC β I to assess PMA potentiation of I_{NMDA} could not be used because this antibody is not specific for PKC β I and exhibits cross-reactivity with PKC α (Popp et al., 2006).

PMA Potentiation of NMDA Receptor Function Is Abolished in PKC Down-Regulated Cells and Cannot Be Rescued by Reintroduction of Catalytically Active PKC β I, β II, or γ into the Cell. Results from the previous experiments provided strong evidence that PMA augmentation of I_{P_k} was mediated only by activation of PKC α . Therefore, we postulated that augmentation of I_{P_k} could be recovered in cells in which all PMA-sensitive PKC isoforms had been down-regulated by addition of PKC α to the inside of the cell during whole-cell patch-clamp experiments. We also predicted that recovery of PMA-mediated augmentation of NMDA-induced currents would be specific for PKC α and that the other classical PKC isoforms would have no effect. To test this postulate, dishes were treated overnight with 100 nM PMA and again for 30 min (ON PMA challenge) before recording. This treatment results in the down-regulation of all PMA-sensitive PKC isoforms (Matthies et al., 1987; Popp et al., 2006). As predicted, there was no increase in I_{P_k} [$F_{(5,42)} = 1.4$; $P = 0.25$] and I_{SS} [$F_{(5,42)} = 0.03$; $P = 0.99$] for up to 20 min, with no significant changes in I_{SS}/I_{P_k} [$F_{(5,42)} = 1.46$; $P = 0.22$] (Fig. 4, A and B). The loss of PMA augmentation of NMDA receptor function was not recovered when catalytically active PKC β I, β II, or γ was introduced into PKC-deficient cells during whole-cell recordings. Dialysis of PKC β I (1 μ g/ml) into the cell did not recover PMA augmentation of I_{P_k} at any time point measured [$F_{(5,42)} = 0.3$; $P = 0.9$]. Changes in I_{SS} [$F_{(5,42)} = 0.16$; $P = 0.9$] and I_{SS}/I_{P_k} [$F_{(5,42)} = 0.48$; $P = 0.78$] were also not observed. Representative current traces and summary graph are shown in Fig. 4, C and D, respectively. When present intracellularly, catalytically active PKC β II (1 μ g/ml) did not enhance I_{P_k} [$F_{(5,42)} = 0.45$; $P = 0.8$], I_{SS} [$F_{(5,42)} = 0.45$; $P = 0.8$], or I_{SS}/I_{P_k} [$F_{(5,42)} = 0.23$; $P = 0.9$]. Similar results were observed when catalytically active PKC γ (1 μ g/ml) was introduced into PMA-sensitive PKC-depleted cells during whole-cell recording [$F_{(5,42)} = 0.7$; $P = 0.6$ for I_{P_k} , $F_{(5,42)} = 0.33$; $P = 0.8$ for I_{SS} , and $F_{(5,42)} = 0.13$; $P = 0.9$ for I_{SS}/I_{P_k}]. Representative current traces and summary graphs are shown in Fig. 4, E and F, for PKC β II and Fig. 4, G and H, and PKC γ . Previous experiments (Chen and Huang, 1991) had indicated that 5 to 7 min was sufficient time for the enzyme to completely dialyze throughout the cell. In our experiments, given the additional 2 to 3 min required to commence the collection of data at time 0, dialysis should have been completed between the 4- and 8-min time points, and we would predict increases in I_{P_k} starting at 4 min, with significant increases at 8 min as observed with the PMA experiments shown in Fig. 1, A and B.

Catalytically Active PKC α Is the Only Classical PKC Isoform That Rescues PMA Potentiation of NMDA Receptor Function in PKC Down-Regulated Cells. To confirm that the PKC enzymes used in our experiments were

Fig. 4. PMA does not potentiate NMDA receptor function in PKC-depleted cells and potentiation is not recovered by the addition of catalytically active PKC β I, β II, or γ into the cell during whole-cell recordings. To eliminate PKC effects on PMA-induced changes in NMDA receptor function, cells were treated overnight with 100 nM PMA and 30 min PMA before recording (ON PMA challenge). This treatment causes the down-regulation of PMA-sensitive PKC isoforms. A, representative current traces taken from a 15 DIV CGC in which PMA did not potentiate I_{P_k} due to the down-regulation of all PMA-sensitive PKC isoforms. Data were obtained from eight different cells and are summarized in B (mean $\pm$ S.E.M.). Adding catalytically active PKC β I into the cell (1 μ g/ml) via the patch electrode did not restore PMA augmentation of I_{P_k} in PKC-depleted cells. C, representative current traces taken from a 15 DIV CGC indicate no PMA-induced increase in I_{P_k} with PKC β I present in the cell. D, summarized data. Similar results were observed when catalytically active PKC β II (1 μ g/ml) and PKC γ (1 μ g/ml) were introduced into the cell during whole-cell recordings. Representative current traces acquired during experiments conducted with PKC β II with summarized data depicting no PMA augmentation of I_{P_k} are shown in E and F, respectively, and current traces and summarized data for recordings with intracellular PKC γ are shown in G and H, respectively. Representative current traces in E and G were obtained from 14 DIV CGCs. All graphs composed of summarized data $\pm$ S.E.M. are from eight cells.

active and to address potential differences in specific activity (provided by the manufacturer), kinase activity assays were conducted on PKC α , β I, β II, and γ . Results from these experiments are shown in Fig. 5 and indicate that the specific activity of PKC α was greater than the specific activity for PKC β I, β II, and γ . To ensure that we were using an equivalent amount of kinase activity, we diluted PKC α 12-fold that resulted in a level of catalytic activity similar to that of PKC β I, β II, and γ . The catalytic activity of the diluted PKC α ,

Fig. 5. Verification of the catalytic activity of PKC isoforms. The specific activity of PKC α , β 1, β 2, and γ purchased from Millipore was assayed using H1 histone as a substrate as described under *Materials and Methods*. Quantification of 32 P incorporation into H1 histone was done using a PhosphorImager. The graph shows relative units of incorporation for 50 ng of each PKC isoform assayed. For PKC α , 3.9 and 50 ng of protein were assayed. The average of three independent measurements is shown.

as well as the specific activities of the other classical isoforms, was similar to the positive control, catalytically active PKC δ . Catalytically active PKC α was introduced into the cell via the patch solution in PKC-depleted cells at three different concentrations based on specific activity: 18-fold greater (1 μ g/ml) and 10- (0.1 μ g/ml) or 20-fold (0.05 μ g/ml) less than the other PKC enzymes. As predicted, dialysis of catalytically active PKC α (1 μ g/ml) significantly enhanced NMDA receptor function [$F_{(5,42)} = 10.0$; $P \leq 0.001$] (Fig. 6A). I_{P_k} significantly increased from 31.27 ± 1.5 pA/pF at 0 min to 38.82 ± 2.0 pA/pF at 4 min ($t = 2.2$, $P \leq 0.05$), 47.66 ± 3.1 pA/pF at 12 min ($t = 4.9$, $P \leq 0.001$), and 50.71 ± 3.1 pA/pF at 20 min ($t = 5.8$, $P \leq 0.001$) (Fig. 6B). Although I_{P_k} increased over the 20 min, this increase was not significant past the 4-min time point ($t = 1.7$, $P \geq 0.05$ between the 4- and 20-min time points). Nonsignificant increases in I_{SS} [$F_{(5,42)} = 0.17$; $P = 0.96$] were observed that also resulted in no change in I_{SS}/I_{P_k} [$F_{(5,42)} = 1.1$; $P = 0.3$]. Introduction of PKC α , diluted to a specific activity comparable with that of PKC β I, β II, and γ (0.1 μ g/ml) and introduced into PKC-depleted cells via the patch electrode during whole-cell recordings also resulted in augmentation of I_{P_k} [$F_{(5,42)} = 10.3$; $P \leq 0.001$] (Fig. 6C). I_{P_k} significantly increased from 41.48 ± 0.7 pA/pF at 0 min to 47.11 ± 1.6 pA/pF at 4 min ($t = 2.4$, $P \leq 0.05$), 52.97 ± 1.7 pA/pF at 12 min ($t = 5.0$, $P \leq 0.001$), and 55.4 ± 2.4 pA/pF at 20 min ($t = 6.1$, $P \leq 0.001$) (Fig. 6D). Increases in I_{P_k} plateaued at 8 min as observed by no further significant increases among the later time points ($t = 2.5$, $P \geq 0.05$ between the 8- and 20-min time points). No significant changes were observed for I_{SS} [$F_{(5,42)} = 0.42$; $P = 0.8$] and I_{SS}/I_{P_k} [$F_{(5,42)} = 0.5$; $P = 0.7$]. Further dilution of PKC α (0.05 μ g/ml) resulted in augmentation of I_{P_k} [$F_{(5,42)} = 5.6$; $P \leq 0.001$] (Fig. 6E) without changing I_{SS} [$F_{(5,42)} = 0.8$; $P = 0.5$] or I_{SS}/I_{P_k} [$F_{(5,42)} = 0.2$; $P = 0.9$]. There was no significant increase in I_{P_k} from 0 to 4 min ($t = 1.4$, $P \geq 0.05$), but I_{P_k} was significantly greater at 12 min (65.51 ± 3.6 pA/pF) and at 20 min (72.2 ± 5.3 pA/pF) compared with the zero time point (49.4 ± 2.1 pA/pF) ($t = 3.2$, $P \leq 0.01$ and $t = 4.5$, $P \leq 0.001$ for 0 to 12 and 0 to 20 min, respectively) (Fig. 6F). Increases in I_{P_k} did not significantly increase after the 8-min time point because this was the last time point at which I_{P_k} significantly differed from values observed at 20 min ($t = 2.27$, $P \leq 0.05$).

Fig. 6. Catalytically active PKC α restores PMA potentiation of NMDA receptor function in PKC-depleted cells. The addition of catalytically active PKC α (1 μ g/ml) into the cell via the patch electrode reversed the effects of the ON PMA challenge treatment described above in that PMA potentiation of I_{PK} was regained. **A**, representative current traces taken from a 14 DIV CGC depicting increasing potentiation of I_{PK} throughout the 20-min recording when PKC α is reintroduced into the cell. Data were obtained from eight different cells and are summarized in **B** (mean $\pm$ S.E.M.). Diluted PKC α (0.1 μ g) that resulted in a specific activity similar to that of the enzymes used in Fig. 4 also resulted in sustained augmentation of I_{PK} . **C**, representative current traces taken from a 15 DIV CGC, and summarized data (mean $\pm$ S.E.M.) are shown in **D**. **E**, representative current traces from a 15 DIV PKC-depleted CGC in which augmentation of I_{PK} was observed when 0.05 μ g/ml catalytically active PKC α was introduced into the cell during whole-cell recording. However, unlike the results obtained with 1 μ g/ml PKC α , I_{PK} did not continue to rise over the 20 min when the two lower concentrations of PKC α were introduced into the PKC-depleted cells. For both 0.1 and 0.05 μ g/ml concentrations of PKC α , I_{PK} significantly increased until 12 min and remained elevated without further increase until termination of the experiments (20 min): $t_{12-20 \text{ min}} = 0.6$; not significant (ns) for PKC α (0.1 μ g/ml) and $t_{12-20 \text{ min}} = 1.3$ for PKC α (0.05 μ g/ml). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

Discussion

The purpose of this study was to identify which of the seven PMA-sensitive PKC isoforms expressed in our primary cultured CGCs was responsible for the potentiating effects of NMDA receptor function by PMA. Our results provide strong evidence that augmentation of NMDA receptor function in primary cultured CGCs 13 to 15 DIV by PMA is due to activation of PKC α and no other PKC isoform. We had reported previously that a 12.5-min, 100 nM PMA exposure at 37°C resulted in significant increases in I_{PK} and I_{SS} , with increases in I_{PK} being time-dependent, whereas increases in I_{SS} were not (Popp et al., 2008a). However, modest increases in I_{SS} over time were sufficient to negate changes in receptor desensitization, a result that differed from previously reported observations (Lu et al., 1999, 2000; Jackson et al., 2006). We attributed these differences to agonist exposure

time: short (Popp et al., 2008a) compared with prolonged (Lu et al., 1999, 2000; Jackson et al., 2006) that could result in glycine-insensitive desensitization. Given that our current results obtained with longer exposure to agonists resulted in only significant augmentation of I_{PK} , it would seem that our previous postulate is true and that PMA changes in I_{SS} can be obscured by agonist-induced desensitization of the receptor. Although most experimental conditions that resulted in significant increases in I_{PK} did not result in a significant change in I_{SS}/I_{PK} , NMDA receptor desensitization (significant decrease in I_{SS}/I_{PK}) was observed when antibodies directed against PKC ϵ and θ were inside the cell, suggesting that the antibodies were blocking the activity of PKC ϵ and θ . However, because I_{PK} continued to increase over time, our interpretation of these results is that these PKC isoforms may have intracellular substrates that can modulate NMDA receptor desensitization but have no effect on the mechanism responsible for PMA increases in I_{PK} . Agonist-induced desensitization of NMDA receptors is a complex phenomenon and specific PKC isoforms may play a role in receptor desensitization either by direct phosphorylation of the receptor or associated proteins or in combination with other intracellular signaling cascades. The changes in I_{SS}/I_{PK} observed when antibodies directed against PKC ϵ and PKC θ were present inside the cell support this statement but are speculative, and confirming a role for novel PKC isoforms in NMDA receptor desensitization is beyond the scope of this article.

Results from the Ca²⁺-free experiments suggested that one of the classical PKC isoforms was responsible for PMA augmentation of I_{PK} . This was further supported by results obtained from experiments in which we used PKC-specific antibodies to block PMA augmentation of I_{PK} . Antibodies that are specific for a given protein have been used previously not only for the identification and determination of the presence of a specific subtype of nACh receptor within a cell ($\alpha 7$) but also to attenuate receptor function due to an allosteric modulator (Cuevas and Berg, 1998; Cuevas et al., 2000). Monoclonal antibodies specific for the $\alpha 7$ -nACh receptor subunit when dialyzed into the cell during whole-cell patch-clamp recordings diminished attenuation by α -bungarotoxin of acetylcholine-mediated currents. It has been argued (Matthies et al., 1987) that PKC antagonists are of low potency and low specificity especially when used in living cells. Results from our laboratory (unpublished data) were also inconclusive when we used commercially available PKC inhibitors. Thus, we used the novel approach of introducing PKC isoform-specific antibodies (Popp et al., 2006) into the cell via the patch electrode to prove our hypothesis that a calcium-specific PKC isoform was responsible for potentiation of I_{PK} . Indeed, intracellular dilution of antibodies directed against the novel non-Ca²⁺-requiring PKC isoforms did not block PMA-mediated potentiation of I_{PK} . Furthermore, potentiation of I_{PK} seemed not to be mediated by all of the classical PKC isoforms but was specific for PKC α . It was only when antibodies directed against PKC α were introduced into the cell that PMA potentiation of I_{PK} was abrogated at all time points. However, when all antibodies were present inside the cell, PMA did not significantly increase I_{PK} at the 4-min time point, a phenomenon observed with PMA alone or with the two higher concentrations of catalytically active PKC α . The mechanism by which anti-PKC α blocks PKC function in our experiments is unknown. In the experiments by Cuevas and

Berg (1998), an exact mechanism by which anti- $\alpha 7$ -nACh blocked α -bungarotoxin modulation of nACh receptors was not determined. Similar to Cuevas and Berg (1998), we can postulate several proposed mechanisms: simple occlusion of enzyme activation sites or the antibody may prevent the conformational change required for the enzyme to unfold and disassociate from the autoinhibitory pseudosubstrate site in the substrate-binding cavity (Newton, 2001), thus prohibiting phosphorylation of the NMDA receptor or associated protein responsible for receptor augmentation. Speculating on the mechanism as to how individual PKC isoform antibodies might be altering the actions of the enzyme inside the cell makes it difficult to explain the changes in NMDA receptor desensitization observed with anti-PKC ϵ and θ as well as explain how all antibodies delayed significant enhancement of I_{PK} . However, despite the delay in significant increases in I_{PK} , I_{PK} did continue to increase over the 20-min recording. Furthermore, maximal I_{PK} values (pA/pF) are similar among the antibody experiments, with maximal I_{PK} values ranging from 67.5 ± 2.8 for anti-PKC θ to 77.7 ± 4.3 for anti-PKC β II, and similar to I_{PK} values (74 ± 2.4 pA/pF) observed for a 30-min, 100 nM PMA exposure at 37°C (Popp et al., 2008a). For most experiments, increases in I_{PK} plateaued after 8 min, and further increases in I_{PK} were not significantly different. This is similar to the results of Chen and Huang (1991, 1992) who reported I_{NMDA} peaked between 7 and 10 min due to intracellular dialysis of PKC.

Because, verification that the antibodies were blocking their respective PKC isoforms inside the cell could not be empirically determined, we conducted experiments in PKC-depleted cells and tried to rescue augmentation of I_{PK} with catalytically active Ca^{2+} -sensitive PKC isoforms. The ability to recover PMA potentiation of I_{PK} in PKC-deficient CGCs with catalytically active PKC α and not with the other PKC classical isoforms further supports the argument that augmentation of NMDA receptor function is mediated by only this PKC isoform. Previous studies have indicated that augmentation of hippocampal NMDA receptors is by activation of the classical PKC isoforms in that the catalytic fragment of PKC that resulted in potentiation of I_{NMDA} during whole-cell patch-clamp experiments was purified from rat brain PKC isoforms α , β , and γ (Xiong et al., 1998). In cortical pyramidal neurons, PKC potentiation of I_{NMDA} (Tyszkiewicz et al., 2004) was determined by an increase in enzyme phosphorylation by a nonspecific phospho-antibody that detected PKC α , β I, β II, ϵ , θ , and δ . The NR1, NR2A, and NR2B NMDA receptor subunits contained in cortex and hippocampal brain regions are phosphorylated by PKC isoforms α , β , and γ (Leonard and Hell, 1997). Phosphorylation of amino acids specific to the first C-terminal exon (C1) of the NMDA NR1 subunit have been shown to be critical for augmentation of NMDA receptor function in *Xenopus* oocytes (Logan et al., 1999). Specific PKC phosphorylation sites Ser890 and Ser896 on the NR1 subunit, first identified by Tingley et al. (1993, 1997), have since been shown to be preferentially phosphorylated by PKC γ and PKC α , respectively (Sánchez-Pérez and Felipe, 2005). Furthermore, phosphorylation of Ser896 results in enhanced trafficking of NMDA receptors to the plasma membrane in hippocampal neurons (Scott et al., 2001). Potentiation of I_{NMDA} observed in the current and previous experiments (Popp et al., 2008a) could be due to an increase in plasma membrane expression of NMDA receptors

resultant of PKC activation, specifically PKC α . Given that NMDA-induced currents are normalized to cell capacitance, this is a viable mechanism.

Collectively, results from these experiments implicate that activation of the classical PKC isoforms results 1) in phosphorylation of specific sites on the NMDA receptor NR1, NR2A, and NR2B subunits; and 2) in augmentation of receptor function. In addition, these data suggest that the classical PKC isoforms mediate effects on NMDA receptors expressed in multiple brain regions. Despite much evidence supporting that NMDA receptor potentiation by PKC activation is by direct phosphorylation of the receptor, not all studies support this premise (Yamakura et al., 1993; Sigel et al., 1994; Zheng et al., 1999). Although our data neither support nor refute that increases in NMDA receptor function caused by activation of PKC α are due to direct phosphorylation of receptor subunits or indirectly by phosphorylation of a protein associated with the receptor, this is the first study to show that a single cellular effect (augmentation of NMDA receptor function) due to PMA exposure is mediated by the activation of one PKC isoform despite the intracellular presence of multiple PMA-sensitive PKC isoforms.

Acknowledgments

We thank Angela Ohm for technical assistance with the PKC activity assays and Dr. David Lovinger for advice and critical evaluation of the manuscript.

References

- Bart AE (1976) Introduction to the analysis of variance, in *Basic Statistical Concepts in Education and the Behavioral Sciences*, p 287, Burgess Publishing Co., Minneapolis, MN.
- Chatterton JE, Awobuluyi M, Premkumar LS, Takahashi H, Talantova M, Shin Y, Cui J, Tu S, Sevarino KA, Nakanishi N, Tong G, Lipton SA, and Zhang D (2002) Excitatory glycine receptors containing the NR3 family of NMDA receptor subunits. *Nature* **415**:793–798.
- Chen L and Huang LY (1991) Sustained potentiation of NMDA receptor-mediated glutamate responses through activation of protein kinase C by a μ opioid. *Neuron* **7**:319–326.
- Chen L and Huang LY (1992) Protein kinase C reduces Mg^{2+} block of NMDA-receptor channels as a mechanism of modulation. *Nature* **356**:521–523.
- Ciabarra AM, Sullivan JM, Gahn LG, Pecht G, Heinemann S, and Sevarino KA (1995) Cloning and characterization of α -1: a developmentally regulated member of a novel class of the ionotropic glutamate receptor family. *J Neurosci* **15**:6498–6508.
- Cuevas J and Berg DK (1998) Mammalian nicotinic receptors with $\alpha 7$ subunits that slowly desensitize and rapidly recover from α -bungarotoxin blockade. *J Neurosci* **18**:10335–10344.
- Cuevas J, Roth AL, and Berg DK (2000) Two distinct classes of functional 7-containing nicotinic receptor on rat superior cervical ganglion neurons. *J Physiol* **525**:735–746.
- Farrant M, Feldmeyer D, Takahashi T, and Cull-Candy SG (1994) NMDA-receptor channel diversity in the developing cerebellum. *Nature* **368**:335–339.
- Gallo V, Kingsbury A, Balázs R, and Jørgensen OS (1987) The role in depolarization in the survival and differentiation of cerebellar granule cells in culture. *J Neurosci* **7**:2203–2213.
- Gerber G, Kangrga I, Ryu PD, Larew JS, and Randic M (1989) Multiple effects of phorbol esters in the rat spinal dorsal horn. *J Neurosci* **9**:3606–3617.
- Grant ER, Bacskai BJ, Aneagawa NJ, Pleasure DE, and Lynch DR (1998) Opposing contributions of NR1 and NR2 to protein kinase C modulation of NMDA receptors. *J Neurochem* **71**:1471–1481.
- Jackson MF, Konarski JZ, Weerapura M, Czerwinski W, and MacDonald JF (2006) Protein kinase C enhances glycine-insensitive desensitization of NMDA receptors independently of previously identified protein kinase C sites. *J Neurochem* **96**:1509–1518.
- Kleckner NW and Dingledine R (1988) Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus* oocytes. *Science* **241**:835–837.
- Lan JY, Skeberdis VA, Jover T, Grooms SY, Lin Y, Araneda RC, Zheng X, Bennett MV, and Zukin RS (2001) Protein kinase C modulates NMDA receptor trafficking and gating. *Nat Neurosci* **4**:382–390.
- Leonard AS and Hell JW (1997) Cyclic AMP-dependent protein kinase and protein kinase C phosphorylate N-methyl-D-aspartate receptors at different sites. *J Biol Chem* **272**:12107–12115.
- Liao GY, Wagner DA, Hsu MH, and Leonard JP (2001) Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. *Mol Pharmacol* **59**:960–964.
- Logan SM, Rivera FE, and Leonard JP (1999) Protein kinase C modulation of

- recombinant NMDA receptor currents: roles for the C-terminal C1 exon and calcium ions. *J Neurosci* **19**:974–986.
- Lu WY, Jackson MF, Bai D, Orser BA, and MacDonald JF (2000) In CA1 pyramidal neurons of the hippocampus protein kinase C regulates calcium-dependent inactivation of NMDA receptors. *J Neurosci* **20**:4452–4461.
- Lu WY, Xiong ZG, Lei S, Orser BA, Dudek E, Browning MD, and MacDonald JF (1999) G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. *Nat Neurosci* **2**:331–338.
- Matthies HJ, Palfrey HC, Hirning LD, and Miller RJ (1987) Down-regulation of protein kinase C in neuronal cells: effects on neurotransmitter release. *J Neurosci* **7**:1198–1206.
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, and Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**:529–540.
- Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, and Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. *Nature* **354**:31–37.
- Newton AC (2001) Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* **101**:2353–2364.
- Ohno S and Nishizuka Y (2002) Protein kinase C isoforms and their specific functions: prologue. *J Biochem* **132**:509–511.
- Popp RL, Lickteig RL, and Lovinger DM (1999) Factors that enhance ethanol inhibition of *N*-methyl-D-aspartate receptors in cerebellar granule cells. *J Pharmacol Exp Ther* **289**:1564–1574.
- Popp RL, Velasquez O, Bland J, and Hughes P (2006) Characterization of protein kinase C isoforms in primary cultured cerebellar granule cells. *Brain Res* **1083**:70–84.
- Popp RL and Dertien JS (2008) Actin depolymerization contributes to ethanol inhibition of NMDA receptors in primary cultured cerebellar granule cells. *Alcohol* **42**:525–539.
- Popp RL, Reneau JC, and Brotherton BJ (2008a) Activation of protein kinase C enhances NMDA-induced currents in primary cultured cerebellar granule cells: effect of temperature and NMDA NR2 subunit composition. *Eur J Pharmacol* **599**:1–10.
- Popp RL, Reneau JC, and Dertien JS (2008b) Cerebellar granule cells cultured from adolescent rats express functional *N*-methyl-D-aspartate receptors: an *in vitro* model for studying the developing cerebellum. *J Neurochem* **106**:900–911.
- Sánchez-Pérez AM and Felipe V (2005) Serines 890 and 896 of the NMDA receptor subunit NR1 are differentially phosphorylated by protein kinase C isoforms. *Neurochem Int* **47**:84–91.
- Scott DB, Blanpied TA, Swanson GT, Zhang C, and Ehlers MD (2001) An NMDA receptor ER retention signal regulated by phosphorylation and alternative splicing. *J Neurosci* **21**:3063–3072.
- Sigel E, Baur R, and Malherbe P (1994) Protein kinase C transiently activates heteromeric *N*-methyl-D-aspartate receptor channels independent of the phosphorylatable C-terminal splice domain and of consensus phosphorylation sites. *J Biol Chem* **269**:8204–8208.
- Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, and Huganir RL (1997) Characterization of protein kinase A and protein kinase C phosphorylation of the *N*-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. *J Biol Chem* **272**:5157–5166.
- Tingley WG, Roche KW, Thompson AK, and Huganir RL (1993) Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain. *Nature* **364**:70–73.
- Tyszkiewicz JP, Gu Z, Wang X, Cai X, and Yan Z (2004) Group II metabotropic glutamate receptors enhance NMDA receptor currents via a protein kinase C-dependent mechanism in pyramidal neurones of rat prefrontal cortex. *J Physiol* **554**:765–777.
- Xiong ZG, Raouf R, Lu WY, Wang LY, Orser BA, Dudek EM, Browning MD, and MacDonald JF (1998) Regulation of *N*-methyl-D-aspartate receptor function by constitutively active protein kinase C. *Mol Pharmacol* **54**:1055–1063.
- Yamakura T, Mori H, Shimoji K, and Mishina M (1993) Phosphorylation of the carboxyl-terminal domain of the $\zeta 1$ subunit is not responsible for potentiation by TPA of the NMDA receptor channel. *Biochem Biophys Res Commun* **196**:1537–1544.
- Zheng X, Zhang L, Wang AP, Bennett MV, and Zukin RS (1997) Ca^{2+} influx amplifies protein kinase C potentiation of recombinant NMDA receptors. *J Neurosci* **17**:8676–8686.
- Zheng X, Zhang L, Wang AP, Bennett MV, and Zukin RS (1999) Protein kinase C potentiation of *N*-methyl-D-aspartate receptor activity is not mediated by phosphorylation of *N*-methyl-D-aspartate receptor subunits. *Proc Natl Acad Sci U S A* **96**:15262–15267.

Address correspondence to: Dr. R. Lisa Popp, Department of Pharmacology and Neuroscience, Texas Tech University Health Sciences Center, 3601 4th St., STOP 6592, Lubbock, TX 79430. E-mail: lisa.popp@ttuhsc.edu