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Regulation of *N*-Methyl-D-Aspartate Receptor Function by Constitutively Active Protein Kinase C

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ABSTRACT

The ability of the constitutively active fragment of protein kinase C (PKM) to modulate *N*-methyl-D-aspartate (NMDA)-activated currents in cultured mouse hippocampal neurons and acutely isolated CA1 hippocampal neurons from postnatal rats was studied using patch-clamp techniques. The responses of two heterodimeric combinations of recombinant NMDA receptors (NR1a/NR2A and NR1a/NR2B) expressed in human embryonic kidney 293 cells were also examined. Intracellular applications of PKM potentiated NMDA-evoked currents in cultured and isolated CA1 hippocampal neurons. This potentiation was observed in the absence or presence of extracellular Ca^{2+} and was prevented by the coapplication of the inhibitory peptide protein kinase inhibitor(19–36). Furthermore, the PKM-induced potentiation was not a consequence of a reduction in the sensitivity of the currents to voltage-dependent blockade by extracellular Mg^{2+}. We also found different sensitivities of the responses of recombinant NMDA receptors to the intracellular application of PKM. Some potentiation was observed with the NR1a/NR2A subunits, but none was observed with the NR1a/NR2B combination. Applications of PKM to inside-out patches taken from cultured neurons increased the probability of channel opening without changing single-channel current amplitudes or channel open times. Thus, the activation of protein kinase C is associated with potentiation of NMDA receptor function in hippocampal neurons largely through an increase in the probability of channel opening.

L-Glutamate is the major excitatory transmitter in the central nervous system, and it activates three subtypes of ionotropic glutamate receptors, namely the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate, NMDA, and kainate receptors. Of these receptors, most NMDA receptors form channels with a substantial permeability to Ca^{2+} (McBain and Mayer, 1994). The influx of Ca^{2+} through these channels stimulates Ca^{2+}-dependent enzymes such as Ca^{2+}-dependent PKC, and activation of this kinase contributes to the induction of long term potentiation in hippocampal neurons (Bliss and Collingridge, 1993). The function of NMDA channels is in turn regulated by both intracellular Ca^{2+} and PKC. For example, NMDA channels are inactivated by the binding of Ca^{2+} and calmodulin directly to sites on the carboxyl terminus of the NR1 subunit of the receptor (Ehlers *et al*., 1996; Hisatsune *et al*., 1997). Also, phorbol esters potentiate NMDA-activated currents recorded in *Xenopus* oocytes expressing either native receptors (Kelso *et al*., 1992) or cloned receptors (Kutsuwada *et al*., 1992; Urushihara *et al*., 1992; Durand *et al*., 1993; Mori *et al*., 1993; Yamakura *et al*., 1993; Wagner and Leonard, 1996; Zheng *et al*., 1997).

The sites of PKC phosphorylation on the carboxyl terminus of NR1a are located in close proximity to sites where calcium and calmodulin bind to induce inactivation (Ehlers *et al*., 1996; Hisatsune *et al*., 1997). Furthermore, it has been proposed that the PKC-induced phosphorylation of the carboxyl terminus of the NR1 subunit enhances NMDA-evoked responses by reducing the affinity of calmodulin for its site (Hisatsune *et al*., 1997). In contrast, intracellular application of the holoenzyme of PKC enhances NMDA-evoked currents in isolated trigeminal neurons by decreasing the voltage-dependent block of NMDA channels by Mg^{2+} (Chen and Huang, 1992). However, Markram and Segal (1992) reported that phorbol esters inhibit NMDA receptor-mediated responses in the CA1 region of the hippocampus. A similar

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PKC, protein kinase C; PKM, constitutively active fragment of protein kinase C; I_{ss}, steady-state current; I_p, peak current; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PKI, protein kinase inhibitor; HEK, human embryonic kidney; ANOVA, analysis of variance.

depression of these currents is observed in cultured hippocampal neurons (Bartlett *et al.*, 1989). Therefore, it is unclear what effect PKC has on NMDA-evoked currents in hippocampal neurons.

The carboxyl terminus of the NR1a subunit is strongly phosphorylated by PKC (Tingley *et al.*, 1993, 1997). However, phosphorylation of this subunit is not well correlated with the phorbol ester-induced potentiation of NMDA-evoked currents. For example, deletion of the carboxyl terminus, or the absence of these phosphorylation sites in some splice variants of this subunit, is paradoxically associated with an even greater phorbol ester-induced enhancement of NMDA-activated currents (Sigel *et al.*, 1994; Zukin and Bennett, 1995). Recent evidence also shows that the phorbol ester-induced potentiation is amplified by the entry of Ca^{2+} through NMDA channels (Zheng *et al.*, 1997).

To explore these issues in more detail in hippocampal neurons, we examined the effects of PKM on NMDA-evoked currents in cultured mouse hippocampal neurons and in acutely isolated CA1 pyramidal neurons taken from slices of postnatal rat hippocampus. The enzymatic activity of this catalytic fragment is independent of intracellular calcium and diacylglycerol, because the regulatory domain of the kinase is absent. We also investigated the role of Mg^{2+} and Ca^{2+} in the PKM-induced enhancement of NMDA-evoked currents in these neurons. Finally, we examined the effects of PKM on currents mediated by NR1a/NR2A and NR1a/NR2B subunit combinations expressed in HEK293 cells.

Materials and Methods

Cultures of fetal hippocampal neurons were prepared according to the previously described techniques (MacDonald *et al.*, 1989). Briefly, timed-pregnant mice were killed by cervical dislocation. Fetuses were removed, and hippocampi were microdissected and placed in cold Hanks' solution. The hippocampi were then mechanically dissociated by trituration and plated in 35-mm, collagen-coated, culture dishes at densities of $<1 \times 10^6$ cells/ml. The cells were grown in dissociated tissue culture using standard techniques. The cultures were used for electrophysiological recordings 12–17 days after plating. The culture medium was replaced with extracellular solution before recording.

Acutely isolated hippocampal neurons were obtained as described previously (Jarvis *et al.*, 1997). Rats (approximately 2 weeks of age) were anesthetized with halothane and killed by decapitation using a guillotine. The whole brain was removed and placed in cold extracellular solution (see below for composition). Hippocampi were then microdissected and cut into 400–500-μm-thick slices by hand, 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 Chemical, St. Louis, MO). All solutions were bubbled with 100% O_2. After incubation with the enzyme, the slices were washed and kept in enzyme-free extracellular solution until they were used. For isolation of CA1 neurons, one or two slices were transferred into a 35-mm culture dish containing 2 ml of extracellular solution, and the dish was placed on the stage of an inverted phase-contrast microscope. Using two fire-polished (round-tipped) glass pipettes, the CA1 region of the hippocampal slices were cut out and mechanically abraded to obtain single cells. Electrophysiological recording of the isolated neurons began approximately 15 min after mechanical dissociation.

HEK293 cells were maintained in a mixture of minimal essential medium with Earle's salts and L-glutamine (Gibco BRL, Gaithersburg, MD), with 10% fetal bovine serum, in a humidified atmosphere containing 5% CO_2. Combinations of NR1a/NR2A and NR1a/NR2B (1:3 ratio, with a total of 2 μg of cDNA/35-mm culture dish) were transfected using the Perfect Lipid method (Invitrogen, Carlsbad, CA). At 24 hr before transfection, cells were plated to a density of approximately 10^6 cells/35-mm dish. After transfection, the cells were cultured in the presence of 1 mM DL-2-amino-5-phosphonovaleric acid. Recordings were made 24–48 hr after transfection.

Patch electrodes were constructed from thin-walled borosilicate glass (1.5-mm diameter; WPI, Sarasota, FL) on a two-stage puller (PP83; Narishige, Tokyo, Japan). The tips of the electrodes were heat-polished on a Narishige microforge (model MF-83; Scientific Instruments Laboratory, Tokyo, Japan) to a final diameter of 1–2 μm. The patch electrodes demonstrated resistances of 3–5 MΩ. Whole-cell currents were recorded using Axopatch 1-D or Axopatch 200A amplifiers (Axon Instruments, Foster City, CA) in the voltage-clamp mode. Data were filtered at 2 kHz and digitized on-line using either the TL-1 or Digidata 1200 DAC units (Axon Instruments). On-line acquisition was performed using pClamp software (Axon

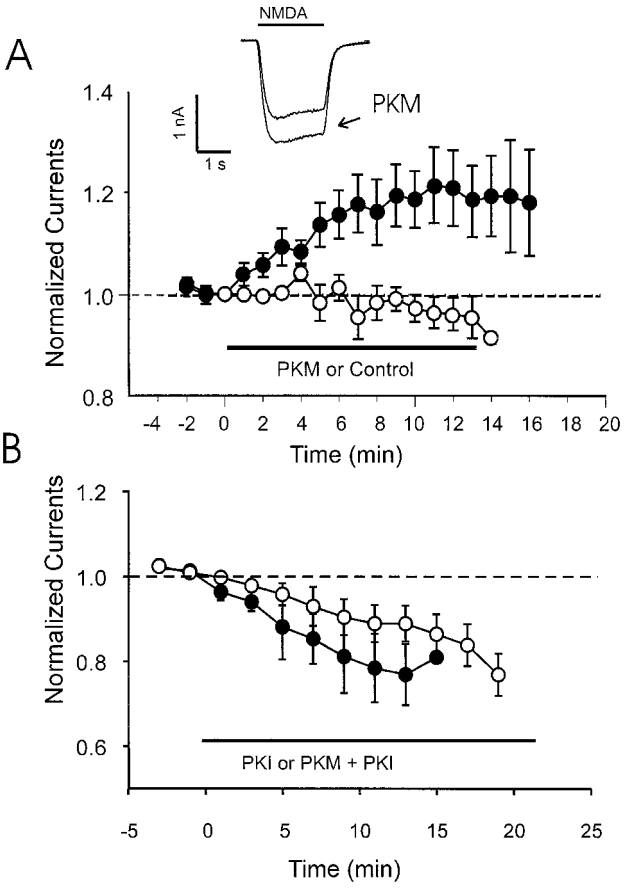

Fig. 1. PKM enhanced NMDA-evoked currents in cultured hippocampal neurons in the presence of 1.3 mM extracellular Ca^{2+}. A, Whole-cell recordings of the peak NMDA-evoked currents (100 μM) are plotted versus the time of the recordings. Cells were perfused either with the dialysis solution used for the preparation of PKM (control) (○) or with PKM (2 μM) (●). *Inset*, sample currents before and after perfusion of PKM; some Ca^{2+}-dependent inactivation is apparent in both traces. The peak responses were normalized to the response before the start of the internal perfusion. NMDA-evoked currents were potentiated by PKM (to 122 ± 5%, $n = 11$, $p < 0.05$, two-factor ANOVA), whereas the control solution was without effect ($n = 6$). B, The responses to NMDA during the intracellular perfusion of a mixture of PKM (2 μM) plus PKI (10 μM) (○) or PKI (10 μM) alone (●) are plotted as described in A. Co-perfusion with PKM and PKI failed to potentiate NMDA-evoked currents ($n = 6$), whereas PKI inhibited these currents ($n = 5$), compared with those recorded after perfusion of the control solution ($p < 0.05$, two-factor ANOVA).

Instruments). In most experiments, the neurons were voltage-clamped at −60 mV. During each experiment, a voltage step of −10 mV was applied before each application of the agonist, to monitor the cell capacitance and the access resistance. Recordings in which the access resistance or the capacitance changed by >10% were not included in data analysis.

The intracellular perfusion was carried out as described previously (Wang *et al*., 1994). Conventional patch electrodes were constructed from 1.5-mm (o.d.), thin-walled, glass tubing (TW 150F-4; WPI) and modified by creating an expansion at the shank near the tip of the electrodes. This expansion allowed for the placement of the tip of the internal pipette closer to the tip of the patch electrode. Internal pipettes were constructed from 0.75-mm (o.d.), thick-walled, glass tubing. The pipettes were pulled on a one-stage electrode puller (model 700C; David Kopf Instruments, Tujunga, CA) with a long taper (1–1.5 cm). The tip of the internal pipette was broken back to a diameter of 10–15 μm. A modified electrode holder was used to hold the internal pipette inside the patch electrode. The position of the internal pipette was adjusted under a microscope to place the tip of the internal pipette within the "bubble" near the tip of the patch electrode (approximately 100 μm from the tip).

Single-channel recordings were made on inside-out patches taken from cultured hippocampal neurons (patch electrodes, 4–8 MΩ). Single-channel events either were captured on-line or were first recorded on video tape using a digital data recorder (VR-10; Instrutech Corp., Mineola, NY) and then acquired using pClamp 6.0 software (Axon Instruments). The single-channel currents were filtered at 2 kHz and sampled at 10 kHz. The records were analyzed using pClamp 6.0 (Axon Instruments).

For whole-cell recordings, the extracellular solution contained 140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 25 mM HEPES, 33 mM glucose, 0.003 mM glycine, and 0.001 mM tetrodotoxin (pH 7.3–7.4, 320–335 mOsM). A multibarrel perfusion system was used to achieve a rapid exchange of NMDA-containing solutions. In some experiments, the solutions containing NMDA (100 μM) lacked added Ca^{2+}, to minimize inactivation of the responses.

For internal perfusion experiments, the pipette solution contained 140 mM KCl, 10 mM HEPES, 5 mM EGTA, 2 mM tetraethylammonium chloride, 1 mM $CaCl_2$, 6 mM $MgCl_2$, and 4 mM K^+-ATP (pH 7.3). Inside-out patch recordings were carried out in solution containing 100 mM K_2SO_4 or Cs_2SO_4, 10 mM HEPES, 1 mM $CaCl_2$, 5 mM EGTA, 2 mM tetraethylammonium chloride, and 4 mM Mg^{2+}-ATP (pH 7.3 at 310 mOsM). The patch pipette was filled with regular extracellular fluid containing 10 μM NMDA and 3 μM glycine.

A mixture of hippocampal PKC isozymes (α, β, and γ) was purified from rat brain as previously described (Roth *et al*., 1989), using sequential chromatography on DEAE cellulose, phenyl-Sepharose, and protamine agarose, and the catalytically active fragment was prepared by digestion with trypsin (Huang and Huang, 1986). The 45-kDa catalytic fragment (PKM) was then re-purified by soybean trypsin inhibitor affinity chromatography. The PKM fractions were pooled, concentrated, and dialyzed extensively against the recording buffer. The samples were then frozen in small aliquots and thawed just before use. The specific activity of the PKM was 1–2 μmol/min/mg, using histone III-S as substrate. The PKM solution was prepared by a 2:1 dilution of the stock solution (PKM dialyzed against regular intracellular solution) with regular intracellular solution containing 8 mM K^+-ATP, to a final concentration of 2 μM PKM and 4 mM K^+-ATP. The stock solution of PKM (50-μl aliquots) was kept at −70° and thawed only before the experiment. To prevent hydrolysis of ATP to ADP, all intracellular solutions containing ATP were kept on ice before the electrodes were filled. PKI (PKC19–36, RFARKGALRQKNVHEVKN; Calbiochem, La Jolla, CA) was used at 10 μM (dissolved in the kinase buffer). In all experiments, the control solution was formulated by adding comparable amounts of the dialysis buffer to the recording solution. All experiments were performed at room temperature (22–24°). Data are expressed as mean ± standard error.

Results

The effects of PKM on NMDA-evoked currents in cultured hippocampal neurons were studied using the internal perfusion technique. Each cell served as its own control (Fig. 1). Neurons were perfused either with a control solution or with solutions containing PKM or the combination of PKM and the inhibitory peptide PKI. Glycine concentrations were maintained at a nearly saturating concentration (3 μM). Nevertheless, responses to NMDA demonstrated some decay during 2-sec applications (Fig. 1A, *inset*), likely resulting from Ca^{2+}-dependent inactivation of the channels. The intracellular perfusion of PKM potentiated NMDA-evoked currents by approximately 20% (to 122 ± 5%, $n = 11$, $p < 0.05$, two-factor ANOVA) (Fig. 1A). In contrast, intracellular perfusion of the control solution into another six cells failed to potentiate these currents (Fig. 1A). When PKM and PKI were co-perfused into a separate series of neurons ($n = 8$), no enhancement of NMDA-evoked currents was observed. Moreover, separate applications of PKI alone ($n = 6$) depressed the NMDA currents from control values (to 84 ± 5%, $p < 0.05$, two-factor ANOVA) (Fig. 1B).

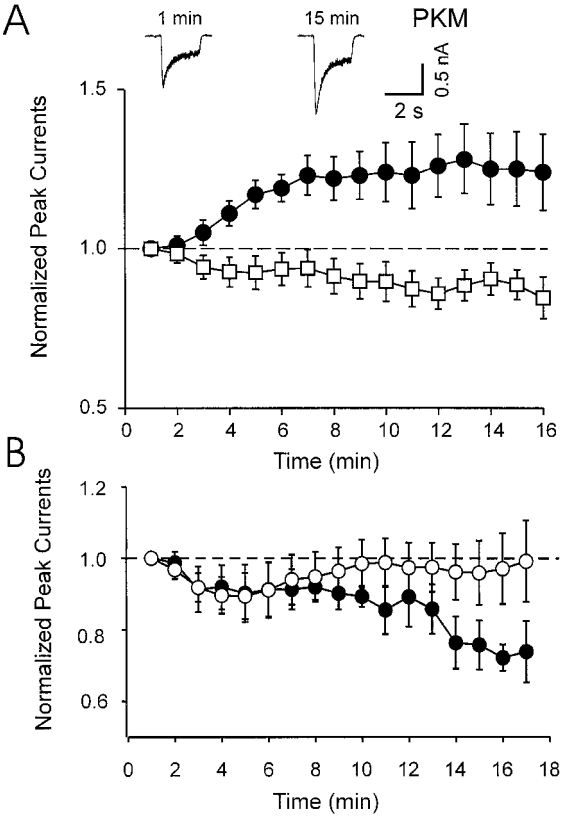

Fig. 2. In acutely isolated CA1 hippocampal neurons, PKM potentiated NMDA-evoked currents. A, Peak NMDA-evoked currents were recorded after breakthrough of the patch. NMDA-activated currents (100 μM NMDA and 3 μM glycine) demonstrated prominent glycine-insensitive desensitization (*inset*). In one series of recordings, the patch pipette contained 1 μM PKM ($n = 15$) or control solution ($n = 14$). Peak currents were normalized to the amplitude of the first current recorded after rupture of the patch. Recordings with PKM-containing patch pipettes (●) showed increases in NMDA-evoked currents to 126 ± 10%, whereas recordings that used the control solution (□) were associated with a decline of currents to 89 ± 5%. B, Another series of recordings ($n = 6$ for each group) were obtained using PKI (4 μM) alone (●) or a mixture of PKM (2 μM) and PKI (4 μM) (○). The presence of PKI prevented the potentiation seen with PKM.

We then examined the effects of PKM on NMDA-evoked currents recorded from isolated CA1 pyramidal neurons. In these isolated neurons, NMDA-evoked currents demonstrated prominent "glycine-independent" desensitization (Fig. 2A, *inset*). A series of recordings was performed with or without PKM (or PKM and PKI) in the patch pipettes. Peak NMDA-evoked currents decreased to approximately 89% of their original amplitudes at breakthrough after 15 min of recording with the control solution in the patch pipette ($n = 14$) (Fig. 2A). In contrast, with PKM in the patch pipette ($n = 15$) peak NMDA-evoked currents increased by approximately 26% (126 ± 10%, $p < 0.001$, two-factor ANOVA) (Fig. 2A). PKM also increased desensitization of NMDA-activated currents (Fig. 2A, *inset*). This was assessed by examining the I_{ss}/I_p ratio within 1 min of the breakthrough and after 15 min of recording. In both PKM and control recordings, this ratio decreased, indicating an increase in desensitization over time. However, the changes in desensitization were larger when PKM was included in the patch pipette (PKM, $I_{ss}/I_p = 0.48 \pm 0.03$ at 1 min, $I_{ss}/I_p = 0.34 \pm 0.02$ at 15 min; control, $I_{ss}/I_p = 0.52 \pm 0.05$ at 1 min, $I_{ss}/I_p = 0.46 \pm 0.03$ at 15 min; $n = 10$ for both groups, $p < 0.05$, two-factor ANOVA). The decreases in the time constants (τ) of desensitization were also consistently greater in the presence of PKM (PKM, approximately 24%, from 575 ± 25 msec to 437 ± 34 msec, $n = 10$; control, approximately 8%, from 589 ± 57 msec to 540 ± 68 msec, $n = 10$; $p < 0.05$, two-factor ANOVA). In a separate series of recordings (Fig. 2B), cells were examined using patch pipettes containing a combination of PKM and PKI ($n = 6$) or PKI by itself ($n = 5$). PKI blocked the potentiation associated with PKM ($p < 0.05$, two-factor ANOVA).

We then examined whether the PKM-induced potentiation of NMDA-evoked currents in cultured or isolated neurons was associated with a change in the sensitivity of the currents to extracellular Mg^{2+}. This was assessed either by combining different concentrations of Mg^{2+} with a fixed concentration of NMDA (i.e., 100 μM) or by applying different concentrations of Mg^{2+} during the application of NMDA (Fig. 3, A and B). Concentration-inhibition relationships for Mg^{2+} were constructed for both isolated and cultured hippocampal

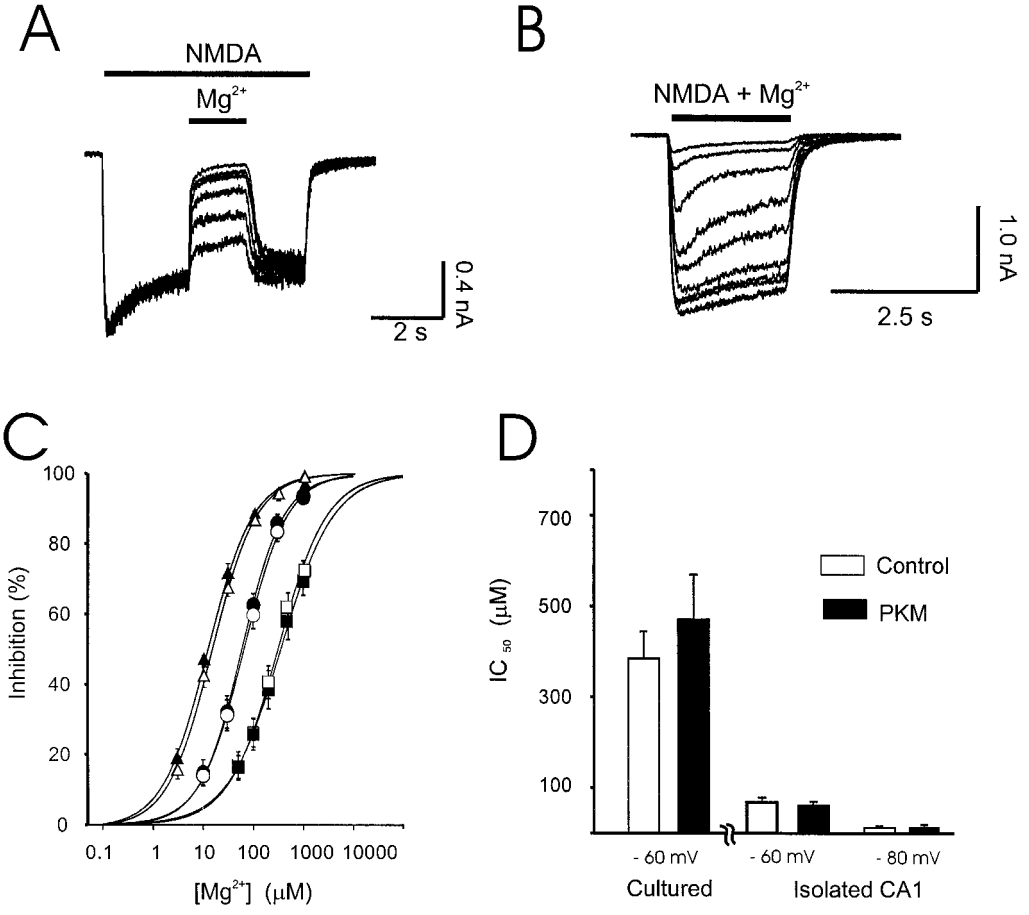

Fig. 3. PKM did not change the sensitivity of cultured and isolated hippocampal neurons to the block by Mg^{2+}, although cultured neurons were markedly less sensitive than isolated neurons. A and B, Representative current traces from cultured neurons for the concentration-response analysis are shown. Mg^{2+} was applied in various concentrations, either simultaneously with NMDA or during application of fixed concentrations of NMDA (50 or 100 μM, with 10 μM glycine). C, Intracellular perfusion of PKM was not associated with a change in the IC_{50} values for the block by Mg^{2+}. The curves were fitted using the relationship $I = I_{max}\{1 - 1/(1 + IC_{50}[Mg^{2+}])^{nH}\}$, where I_{max} is the NMDA-evoked current with 0 mM external Mg^{2+} (no blockade), IC_{50} is the concentration of Mg^{2+} that blocks I_{max} by 50%, and n_H is the Hill coefficient. In cultured neurons held at −60 mV (*squares*), the IC_{50} value was 498 ± 180 μM before (*open symbols*) and 519 ± 183 μM during the application of PKM (*closed symbols*). The Mg^{2+} sensitivity of NMDA currents in acutely isolated neurons held at −60 (*circles*) and −80 mV (*triangles*) also was not changed by the inclusion of PKM in the patch pipettes (control, −60 mV, IC_{50} = 69 ± 12 μM; PKM, −60 mV, 60 ± 8 μM; $n = 6$ each group, $p > 0.05$, Student's t test; control, −80 mV, IC_{50} = 12.2 ± 2.5 μM, $n = 7$; PKM, −80 mV, 14.9 ± 1.6 μM, $n = 11$; $p > 0.05$, Student's t test). D, The IC_{50} values for the Mg^{2+} block of NMDA-evoked currents in both cultured (−60 mV) and acutely dissociated (−60 and −80 mV) hippocampal CA1 neurons were determined. The sensitivity of NMDA-evoked currents to the Mg^{2+} block was significantly higher in dissociated CA1 neurons than in cultured hippocampal neurons ($p < 0.01$, Students t test).

neurons (Fig. 3C), with or without PKM in the patch pipette or after intracellular perfusion, respectively. Applications of PKM did not alter the IC_{50} values for the block by Mg^{2+} in either cultured neurons (control, 498 ± 180 μM; PKM, 519 ± 183 μM; $n = 7$, $p > 0.05$, −60 mV) or isolated neurons (control, 69 ± 12 μM; PKM, 60 ± 8 μM; $n = 6$ each group, $p > 0.05$, −60 mV) (Fig. 3, C and D). In acutely dissociated hippocampal neurons, we also tested the Mg^{2+} sensitivity of NMDA-evoked currents at a more hyperpolarized potential (−80 mV). The sensitivity to the Mg^{2+} block at −80 mV (IC_{50} = 12.2 ± 2.5 μM, $n = 7$) was 5-fold higher than at −60 mV (Fig. 3, C and D). Nevertheless, the presence of PKM in the pipette did not change the IC_{50} of the Mg^{2+} block (IC_{50} = 14.9 ± 1.6 μM, $n = 11$, $p > 0.05$, Student's t test).

We also examined the effects of PKM on the voltage dependence of the Mg^{2+} block of NMDA-activated currents in isolated CA1 neurons. To study a full range of potentials, we used voltage ramps (−100 to +60 mV) (Fig. 4). In some cells, we compared responses to ramps in the absence or presence of Mg^{2+} (100 μM) before (just after breakthrough) and then after the current increase associated with the intracellular application of PKM (15 min) (Fig. 4A). Such currents displayed nearly linear current-voltage relationships in the absence of added extracellular Mg^{2+}, whereas a prominent voltage-dependent block (−100 to approximately −30 mV) was observed with 100 μM Mg^{2+}. The PKM-induced potentiation of NMDA-evoked currents did not appear to be accompanied by any obvious change in the voltage dependence of the Mg^{2+} block. To examine this question more quantitatively, we also compared two groups of neurons, one recorded using the control solution and the other recorded using PKM. NMDA-evoked currents were normalized to those recorded at −40 mV in the absence of Mg^{2+}. There was no change in the voltage dependence of Mg^{2+} block regardless of the value of the membrane potential (Fig. 4, B and C).

Note that cultured neurons demonstrated a wide range of sensitivities to Mg^{2+} and were much less sensitive to the block by Mg^{2+} than were isolated neurons (Fig. 3, C and D) (cultured, IC_{50} = 489 ± 180 μM, $n = 7$; isolated, IC_{50} = 69 ± 12 μM, $n = 6$; −60 mV, $p < 0.001$, Student's t test). In

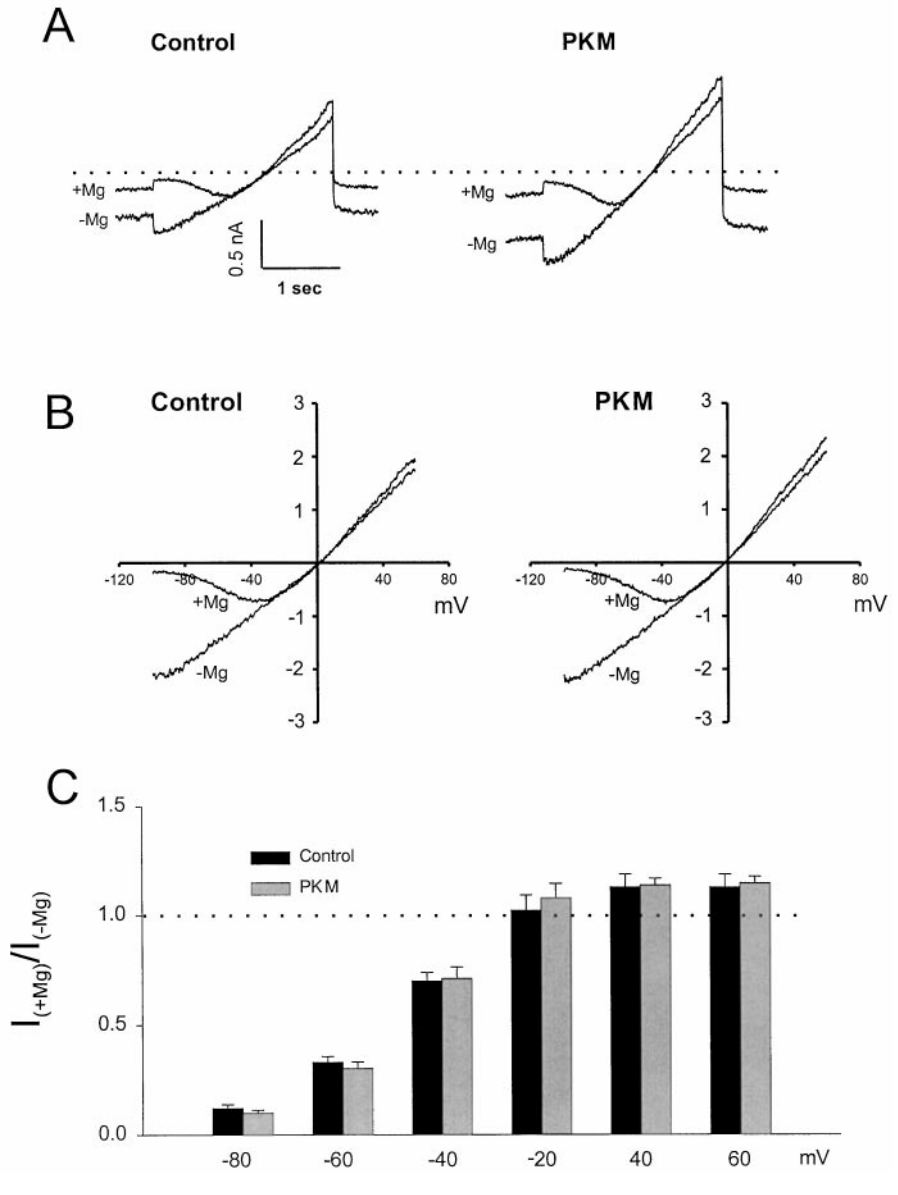

Fig. 4. PKM did not affect the voltage dependence of the Mg^{2+} block of NMDA-evoked currents in isolated CA1 neurons. Responses were activated using 100 μM NMDA and 3 μM glycine, with or without 100 μM Mg^{2+}, at a holding potential of −60 mV. When the current reached a steady state (~3 sec after the beginning of the application), a voltage ramp (2.5 sec) from −100 to +60 mV was applied to the cell. The current generated by a control ramp without NMDA application was used for leak subtraction. A, Sample traces at different times taken from the same cell recorded with intracellular solution containing PKM (control, 1 min after breakthrough of the patch; PKM, 15 min after breakthrough). PKM potentiated NMDA-evoked current at both negative and positive membrane potentials, either with ($+Mg^{2+}$) or without ($-Mg^{2+}$) Mg^{2+}. B, Average current-voltage curves for control and PKM-treated groups ($n = 6$ for each group), in the absence and presence of 100 μM Mg^{2+}. The current amplitudes at different potentials were normalized to that at −40 mV in the absence of added Mg^{2+}. PKM did not change the shape of current-voltage curves before or after Mg^{2+}, indicating that it did not affect the voltage dependence of the Mg^{2+} block of NMDA-activated currents. C, Bar graph showing the effect of Mg^{2+} (100 μM) on NMDA currents at different potentials in both control and PKM-treated groups ($n = 6$ for each group). No difference in the degree of blockade by Mg^{2+} was observed in the presence of PKM.

cultured neurons, an influx of Ca^{2+} through NMDA channels inactivates NMDA-evoked currents even when EGTA is included in the patch pipette (Legendre *et al.*, 1993). In contrast, use of BAPTA, a much more rapid buffer than EGTA, blocks this Ca^{2+} inactivation. To determine whether Ca^{2+} inactivation might have been responsible for the low Mg^{2+} sensitivity of currents in cultured neurons, we compared recordings using BAPTA with those using EGTA (Fig. 5A). The concentration-inhibition relationships for Mg^{2+} block were the same regardless of which buffer was used (EGTA, IC_{50} = 304.2 ± 99 μM; BAPTA, 244.8 ± 63 μM; n = 6 for both groups, $p > 0.05$, Student's t test) (Fig. 5A), suggesting that Ca^{2+} inactivation did not account for the low Mg^{2+} sensitivity of cultured neurons.

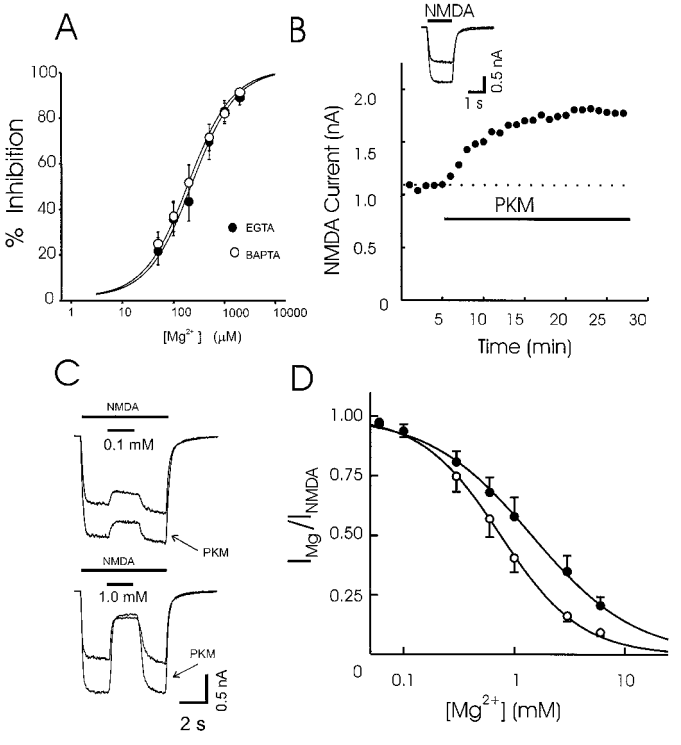

Fig. 5. Ca^{2+} inactivation did not change the Mg^{2+} sensitivity of the NMDA-evoked currents, and intracellular perfusion of PKM potentiated whole-cell currents evoked by NMDA in cultured hippocampal neurons in the absence of extracellular Ca^{2+}. A, The use of EGTA or BAPTA did not change the Mg^{2+} sensitivity of the response to NMDA. Plots of the concentration-inhibition relationships assessed using either EGTA or BAPTA in the patch pipette are shown (n = 6 for each group). The extent of intracellular Ca^{2+} buffering did not significantly affect the Mg^{2+} sensitivity of the NMDA-evoked currents (IC_{50} = 304.2 ± 99 μM with EGTA and 244.8 ± 63 μM with BAPTA, $p > 0.05$, Student's t test). B, In a sample recording obtained in the absence of extracellular Ca^{2+}, perfusion of PKM caused approximately 70% potentiation of the current (before and after 20 min of perfusion). Unlike responses in the presence of Ca^{2+}, no decay of the current was observed during a 2-sec application of NMDA. The enhancement by PKM was gradual in onset, and in a series of 11 neurons PKM enhanced NMDA-activated currents in each of these cells, to a mean value of 154 ± 7.3% of the amplitudes recorded before perfusion (not shown). *C*, The sensitivity of the currents to the block by Mg^{2+} was also assessed using coapplication of a range of concentrations of Mg^{2+} (0.06–6 mM) during the response to NMDA. Sample responses to 0.1 and 1.0 mM Mg^{2+} are shown. D, The concentration-inhibition relationships for Mg^{2+} before (●) and after (○) intracellular perfusion of PKM are plotted for recordings from five cultured neurons. Currents were normalized as the ratio of the current in the presence of Mg^{2+} to that recorded in the absence of a given concentration of this divalent cation (I_{Mg}/I_{NMDA}). The calculated IC_{50} values were 1.45 mM before and 0.76 mM after the perfusion of PKM.

It is also possible that PKM reduced the Ca^{2+} inactivation of NMDA channels, thus potentiating NMDA-evoked currents. Therefore, we repeated the intracellular perfusion of PKM into cultured neurons while applying NMDA in the absence of extracellular Ca^{2+}. This protocol eliminates Ca^{2+} inactivation but also activates a nonselective cation current in cultured hippocampal neurons (Xiong *et al.*, 1997) that contaminates the NMDA-evoked current. The nonselective cation current is also blocked, although less potently than NMDA-activated currents, by extracellular Mg^{2+}, but it is not potentiated by the intracellular perfusion of PKM (Xiong Z-G and MacDonald JF, unpublished observations). Under these recording conditions, the perfusion of PKM was even more effective at potentiating NMDA-evoked currents (158 ± 7%, n = 11, $p < 0.01$, two-factor ANOVA) (Fig. 5B). This enhancement was prevented by the co-perfusion of PKI (data not shown, n = 6). We also tested the sensitivity of the NMDA responses to a block by extracellular Mg^{2+}, both before and after the intracellular perfusion of PKM (Fig. 5, C and D). The concentration-inhibition relationships for Mg^{2+} were constructed from observations made in each cell before and after application of PKM (Fig. 5D). Unexpectedly, the block by Mg^{2+} appeared to be proportionately greater after the application of PKM (before PKM, IC_{50} = 1.45 mM; after PKM, IC_{50} = 0.76 mM), although we attribute this effect to the presence of a relatively Mg^{2+}-insensitive nonselective cation current.

To further examine the mechanism of PKM potentiation of NMDA-evoked currents, we recorded single-channel activity from inside-out patches excised from cultured hippocampal neurons. PKM or PKI and PKM were dissolved in the intracellular solution containing 4 mM ATP and 6 mM Mg^{2+} and were applied to the cytoplasmic side of the patch. The patch pipette contained a relatively low concentration of NMDA (10 μM), to minimize desensitization, and also contained nearly saturating concentrations of glycine (3 μM) but no added Mg^{2+}. The cytoplasmic side of each patch was continuously

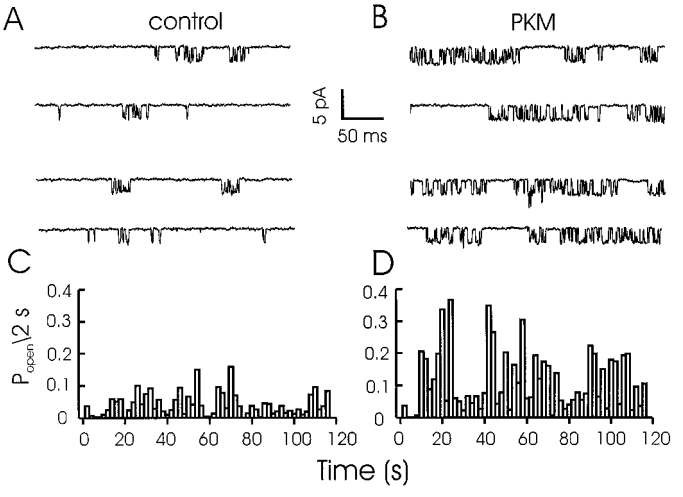

Fig. 6. PKM enhanced NMDA channel activity in inside-out patches taken from cultured hippocampal neurons. A and B, Representative single-channel currents recorded before (A) and during (B) the application of 0.8 μM PKM to the cytoplasmic face of the patch. The channel conductance was not affected by PKM. C and D, Continuous records of NMDA channel open probability (P_{open}) before (C) and during (D) the application of PKM. The mean open probability of the channel was 0.043 before and 0.100 during application of PKM. The holding potential for the inside-out patch was −80 mV (+80-mV pipette potential).

superfused with the intracellular solution. Under these recording conditions, NMDA channels exhibited a single-channel conductance of approximately 40 pS (patch held at −80-mV or +80-mV pipette potential). Applications of PKM (0.8 μM) to the cytoplasmic side of the patch (Fig. 6) increased the open-channel probability to 271% (control, open-channel probability = 0.021 ± 0.008; PKM, open-channel probability = 0.057 ± 0.020; $n = 7$, $p < 0.05$, Wilcoxon signed rank test) without changing the channel conductance (control, 37.8 ± 2.5 pS; PKM, 38.6 ± 3.0 pS; $n = 7$, $p > 0.05$). The open-time histograms were well fit with two exponential functions, and the time constants (τ_1 and τ_2) were not altered by the application of PKM (before PKM, $\tau_1 = 0.38 \pm 0.07$ and $\tau_2 = 2.19 \pm 0.47$ msec; after PKM, $\tau_1 = 0.41 \pm 0.14$ and $\tau_2 = 2.06 \pm 0.64$ msec; $n = 4$, $p > 0.05$, Mann-Whitney U test) (Fig. 7). The closed-time histograms were fit with four components and the τ_4 was significantly reduced after application of PKM, reflecting the increase in the open probability (before PKM, $\tau_4 = 248.75 \pm 30.15$ msec; after PKM, $\tau_4 = 130.67 \pm 5.88$ msec; $n = 4$, $p < 0.05$) (Fig. 7). Coapplications of PKM and PKI did not have any discernible effects on the channel activity recorded ($n = 3$, data not shown).

Native receptors in hippocampal neurons are likely composed of NR1a and NR2A or NR2B subunits. Therefore, we expressed the NR1a/NR2A and NR1a/NR2B subunits in HEK293 cells and recorded with pipettes containing the control solution or PKM. Recording conditions were identical to those used for cultured and isolated neurons. NMDA-evoked currents declined or ran down over a period of 15 min. However, PKM potentiated, or at least slowed, the decrease of responses of the NR1a/NR2A combination (Fig. 8A). At 4 min of whole-cell recording, the I_p amplitude in the control group decreased to 85 ± 6% ($n = 8$) of the initial value, whereas the amplitude in the PKM-treated group increased to 106 ± 9% ($n = 13$) of the initial value (Fig. 8A). Similarly, at 10 min of recording, the amplitude in the control group decreased to 79 ± 5%, whereas the amplitude in the PKM-treated group was 101 ± 12% of the initial value ($p < 0.01$ between the two groups, two-factor ANOVA). In contrast, PKM did not have any effect on the responses of the NR1a/NR2B combination of subunits (Fig. 8B). The effect of PKM on the NR1a/NR2A subunit combination was absent when the pipette also included PKI ($n = 5$, data not shown).

Discussion

Intracellular applications of PKM to both cultured and isolated CA1 hippocampal neurons potentiated NMDA-evoked currents. It is possible that the catalytic fragment had access to additional substrates not readily available to the membrane-bound holoenzyme or that it might have an altered specificity for substrate. However, the latter seems

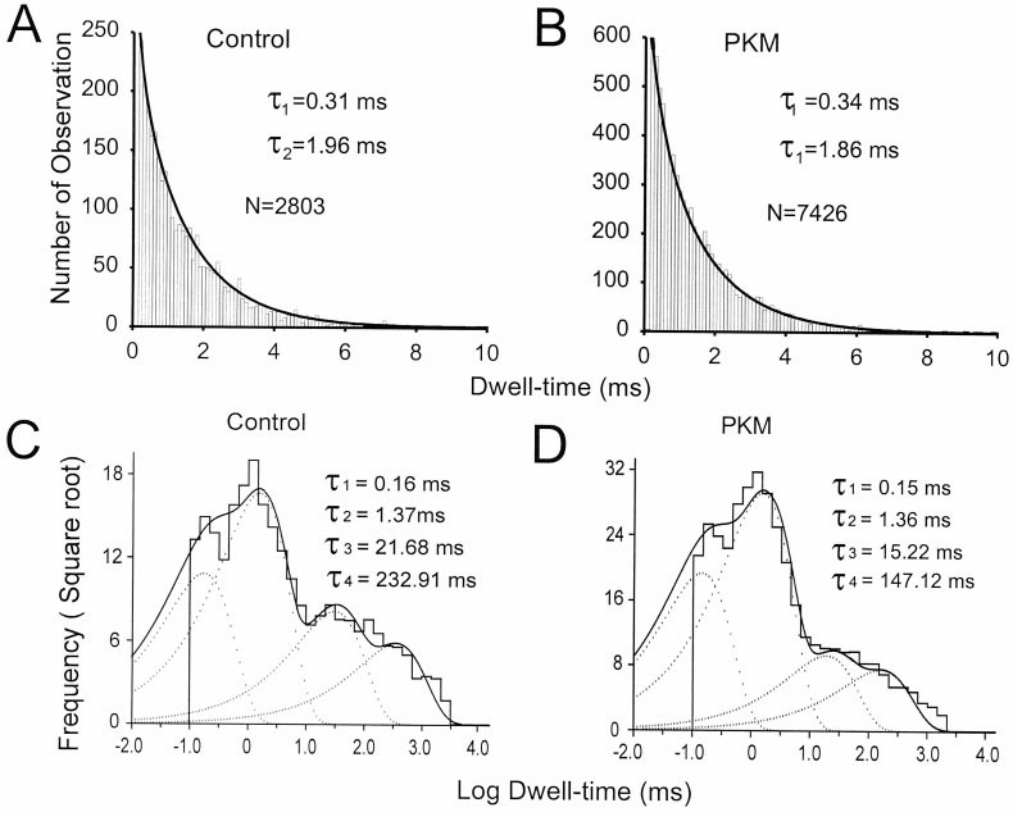

Fig. 7. The effects of PKM on open- and closed-time histograms were determined. A and B, Representative dwell-time histograms of open times before (A) and during (B) application of PKM are shown, and the distributions were fitted with two components. The time constants for the fits are given in the diagram. *Solid line*, sum of the components; *N*, number of events. Application of PKM did not significantly affect the distribution of open times (before PKM, $\tau_1 = 0.38 \pm 0.07$ msec and $\tau_2 = 2.19 \pm 0.47$ msec; after PKM, $\tau_1 = 0.41 \pm 0.14$ msec and $\tau_2 = 2.06 \pm 0.64$ msec; $p > 0.05$, $n = 4$, Mann-Whitney U test). C and D, The distribution of closed times before (C) and during (D) application of PKM are given and were fitted with four components. The long closed time (τ_4) was significantly reduced by application of PKM (before PKM, $\tau_1 = 0.19 \pm 0.06$ msec, $\tau_2 = 1.35 \pm 0.06$ msec, $\tau_3 = 24.3 \pm 5.44$ msec, and $\tau_4 = 248.75 \pm 30.15$ msec; after PKM, $\tau_1 = 0.17 \pm 0.04$ msec, $\tau_2 = 1.49 \pm 0.12$ msec, $\tau_3 = 15.49 \pm 1.37$ msec, and $\tau_4 = 130.67 \pm 5.88$ msec; $n = 4$, $p < 0.05$, Mann-Whitney U test).

unlikely, because the potentiation was blocked by the coperfusion of PKI. Applications of this inhibitory peptide by itself also reduced the amplitude of control currents in cultured neurons, suggesting that some degree of basal phosphorylation was also present.

The potentiation we observed in cultured and isolated neurons was not associated with any change in the sensitivity to Mg^{2+}. The approximately 20–25% potentiation of NMDA-evoked currents observed by us in hippocampal neurons compares favorably with the approximately 20% potentiation (after the intracellular application of the holoenzyme PKC into isolated trigeminal neurons) reported by Chen and Huang (1992) in the absence of extracellular Mg^{2+}. In contrast, we could not detect any change in the voltage dependence of the Mg^{2+} blockade after intracellular perfusion of PKM into either cultured or isolated hippocampal neurons. In addition to the difference in cell types, it was necessary for Chen and Huang (1992) to raise intracellular concentrations of Ca^{2+} and to apply phospholipids to activate the kinase. In contrast, our use of PKM abrogated the requirement for manipulation of the concentrations of intracellular Ca^{2+}, suggesting that alterations in intracellular Ca^{2+} concentrations might have accounted for the discrepancy between their results and our own. For example, the coincident stimulation of PKC and calcium/calmodulin-dependent kinases or the phosphatase calcineurin (Lieberman and Mody, 1994) might have produced more complex effects on NMDA receptor function.

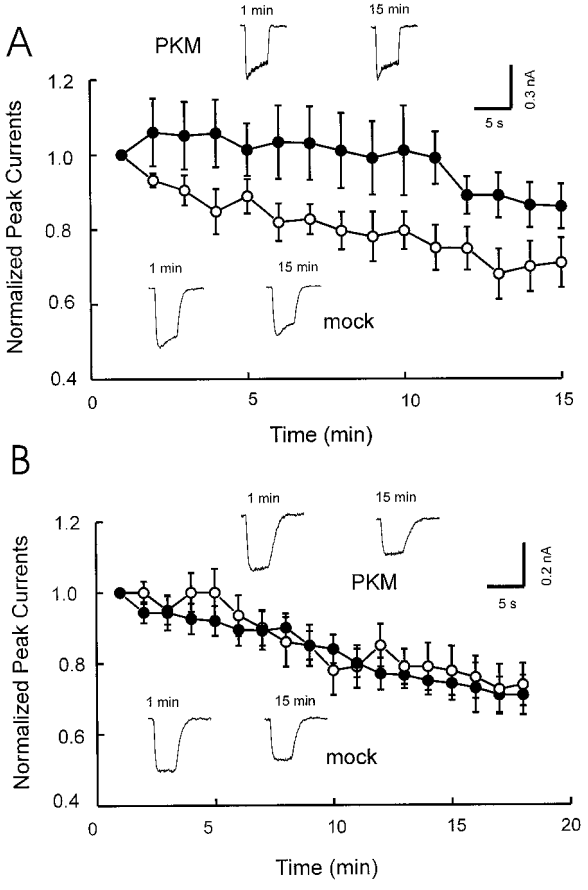

Fig. 8. PKM reduced the run-down of NMDA-evoked currents recorded from HEK293 cells expressing the NR1a/NR2A but not the NR1a/NR2B combination of subunits. A, Responses from a series of cells expressing the NR1a/NR2A combination were recorded with either PKM ($n = 8$) (●) or the control solution ($n = 13$) (○) added to the patch pipette. PKM enhanced currents over the control values for the NR1a/NR2A combination ($p < 0.05$, two-factor ANOVA). *Insets*, sample responses recorded using PKM or control solution (*mock*) in the recording electrode. B, Similar recordings for cells expressing the NR1a/NR2B combination are shown. The control series ($n = 11$) (○) were indistinguishable from recordings in which PKM was used ($n = 12$) (●). NMDA-activated currents (100 μM NMDA and 10 μM glycine) were normalized to the amplitude of the first response in each cell. *Insets*, sample responses recorded using PKM or control solution in the recording electrode.

The phorbol ester-induced potentiation of responses to recombinant NMDA receptors expressed in oocytes also depends in part upon the entry of Ca^{2+} through NMDA channels (Zheng *et al.*, 1997). Therefore, additional Ca^{2+}-dependent processes may amplify the PKC-induced potentiation of NMDA-evoked currents. One possible mechanism for such amplification is a PKC-dependent relief of the Ca^{2+}-dependent inactivation of NMDA channels (Hisatsune *et al.*, 1997). However, this seems unlikely to account for the potentiation observed in hippocampal neurons, because the removal of extracellular Ca^{2+}, and hence the loss of Ca^{2+} inactivation, failed to block the PKM-induced potentiation.

Our results demonstrating little change in the sensitivity to Mg^{2+} are consistent with observations made by Wagner and Leonard (1996). Those authors examined the effects of phorbol esters on NMDA responses mediated by various heterodimeric combinations of NMDA receptor subunits expressed in *Xenopus* oocytes. Although some small shifts in the sensitivity of several of the combinations were observed with very low concentrations of Mg^{2+} (i.e., $\zeta 1/\epsilon 1$, from 2.8 to 6.7 μM), there was no change in the phorbol ester-induced potentiation in the presence of millimolar concentrations of extracellular Mg^{2+}. In agreement with this latter result, we observed no difference in the degree of potentiation of NMDA-activated currents in the absence or presence of a wide range of Mg^{2+} concentrations.

In cultured neurons, the IC_{50} value for the block by Mg^{2+} was much higher than that determined for isolated CA1 neurons ($IC_{50} = 69$ μM, -60 mV). In isolated trigeminal neurons, the apparent affinities for Mg^{2+} are even lower than this (at -60 mV, the Mg^{2+} K_d is 27.3 μM in the absence of PKC and 70.2 μM in PKC-treated cells) (Chen and Huang, 1992). This presents a paradox, wherein whole-cell recordings from cultured neurons demonstrate an approximately 5-fold lower sensitivity to Mg^{2+}, compared with recordings from isolated neurons. This evidence suggests that cultured neurons might express a relatively Mg^{2+}-insensitive combination of receptor subunits. For example, the $\epsilon 3/\zeta 1$ combination is strongly resistant to the block by 1 mM Mg^{2+} (Kutsuwada *et al.*, 1992). Alternatively, the discrepancy might arise because of the difficulties of achieving an adequate space-clamp of large cultured neurons.

The magnitude of the enhancement by PKM in cultured hippocampal neurons is much less than that reported for the effects of phorbol esters on NMDA-activated currents expressed in oocytes. For example, typical values range from 2-fold for native NMDA receptors (Kelso *et al.*, 1992) to 3–4-fold for $\epsilon 2/\zeta 1$ and $\epsilon 1/\zeta 1$ subunit combinations (Kutsuwada *et al.*, 1992) to 3–20-fold for splice variants of NMDAR1 (Durand *et al.*, 1993). However, these large phorbol ester-induced potentiations of NMDA responses may be unrelated to direct phosphorylation of the receptor. For example, treatment of oocytes with cytocalasin D, an agent that disrupts the cy-

toskeleton, reduced the phorbol ester-induced potentiation, suggesting that phosphorylation of cytoskeletal proteins was responsible for an indirect effect on the function of NMDA channels (Wagner and Leonard, 1994). Indeed, evidence suggests that the activity of the NMDA receptor in neurons is also regulated by interactions with cytoskeletal proteins (Rosenmund and Westbrook, 1993; Paoletti and Ascher, 1994) that are themselves potentially subject to phosphorylation by PKC.

The NR2A and NR2B subunits are the major NR2 subunits expressed in hippocampal neurons, and both subunits are phosphorylated by PKC (Leonard and Hell, 1997). For this reason, we examined effects of PKM on the heterodimeric NR1a/NR2A and NR1a/NR2B combinations of subunits in HEK cells. Surprisingly, only the responses of the NR1a/NR2A subunit combination were modified by intracellular applications of PKM. This result suggests that it is phosphorylation of the NR2A subunit or an associated protein and not the NR1a subunit that determines the PKC-induced potentiation of NMDA-evoked currents. A similar and selective enhancement of the NR1/NR2A combination was observed for the potentiation of NMDA responses by the tyrosine kinases $pp60^{c\text{-}src}$ (Src) and $pp55^{fyn}$ (Kohr and Seeburg, 1996), even though the NR2B subunit is reported to be the major tyrosine-phosphorylated protein in the postsynaptic density (Moon *et al*., 1994). Activation of PKC can itself stimulate tyrosine kinases such as Src (Della *et al*., 1997), and Src has been shown to enhance NMDA channel activity in dorsal horn neurons (Yu *et al*., 1997).

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