

Nefiracetam Potentiates *N*-Methyl-D-aspartate (NMDA) Receptor Function via Protein Kinase C Activation and Reduces Magnesium Block of NMDA Receptor

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ABSTRACT

Nicotinic acetylcholine receptors and *N*-methyl-D-aspartate (NMDA) receptors are known to be down-regulated in the brain of Alzheimer's disease patients. We have previously demonstrated that the nootropic drug nefiracetam potentiates the activity of both nicotinic acetylcholine and NMDA receptors and that nefiracetam modulates the glycine binding site of the NMDA receptor. Because the NMDA receptor is also modulated by Mg^{2+} and protein kinases, we studied their roles in nefiracetam action on the NMDA receptor by the whole-cell patch-clamp technique and immunoblotting analysis using rat cortical or hippocampal neurons in primary culture. The nefiracetam potentiation of NMDA currents was inhibited by the protein kinase C (PKC) inhibitor chelerythrine, but not by the protein kinase A (PKA) inhibitor *N*-[2-(4-bromocinnamylamino)ethyl]-5-

isoquinoline (H89). In immunoblotting analysis, nefiracetam treatment increased the PKC α activity with a bell-shaped dose-response relationship peaking at 10 nM, thereby increasing phosphorylation of PKC substrate and NMDA receptor. Such an increase in PKC α -mediated phosphorylation was prevented by chelerythrine. Nefiracetam treatment did not affect the PKA activity. Analysis of the current-voltage relationships revealed that nefiracetam at 10 nM largely eliminated voltage-dependent Mg^{2+} block and that this action of nefiracetam was sensitive to PKC inhibition. It was concluded that nefiracetam potentiated NMDA currents not by acting as a partial agonist but by interacting with PKC, allosterically enhancing glycine binding, and attenuating voltage-dependent Mg^{2+} block.

It is well known that Alzheimer's disease is associated with down-regulation of the cholinergic system in the brain (Giacobini, 2000). Thus, stimulation of the cholinergic system may improve the patient's cognition, learning, and memory. Along this line of thinking, four anticholinesterases have been approved in the United States to improve the conditions of Alzheimer's disease patients. However, these drugs are far from ideal because of their side effects associated with cholinesterase inhibition and a limited period of effectiveness. Therefore, alternative approaches have been sought. Nefiracetam, a pyrrolidone nootropic drug that does not inhibit cholinesterase, was found to improve cognitive function in a

variety of animal models. For example, nefiracetam reduced the amnesia produced by scopolamine (Sakurai et al., 1989) and apomorphine (Nabeshima et al., 1994), and it improved the Morris water maze performance after traumatic brain injury in rats (DeFord et al., 2001). In *in vitro* studies, nefiracetam was found to potentiate the activity of high-voltage-gated N/L-type calcium channels (Yoshii and Watabe, 1994; Yoshii et al., 2000) and $\alpha 4\beta 2$ -type nicotinic acetylcholine (nACh) receptors (Zhao et al., 2001).

It is also known that the glutamatergic system is down-regulated in the brain of Alzheimer's disease patients (Fonnum et al., 1995). Glutamate is the major excitatory neurotransmitter in the central nervous system, and it activates three glutamate ionotropic receptor subtypes: α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, kainate, and *N*-methyl-D-aspartate (NMDA) (Ozawa et al., 1998; Dingledine

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ABBREVIATIONS: nACh, nicotinic acetylcholine; NMDA, *N*-methyl-D-aspartate; I-V, current-voltage; DARPP, dopamine and cAMP-regulated phosphoprotein of 32 kDa; PKC, protein kinase C; PKA, protein kinase A; DM-9384, *N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide; MARCKS, myristoylated alanine-rich C kinase substrate; GluR, glutamate receptor; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; pPKC, phosphorylated protein kinase C; pNR1, phosphorylated NR1.

et al., 1999). The NMDA receptor is implicated in complex neuronal functions such as learning/memory and synaptic plasticity (Collingridge and Singer, 1990; Monyer et al., 1992). The influx of Ca^{2+} ions into neurons after activation of the NMDA receptor is thought to cause modulation of a host of physiological processes, including the induction of synaptic plasticity (Bliss and Collingridge, 1993).

We reported previously that nefiracetam potently augmented NMDA-evoked currents in rat cortical neurons. This action seemed to be exerted via an interaction with the glycine binding site on the NMDA receptor (Moriguchi et al., 2003). Thus, nefiracetam may improve cognitive function by increasing the activity of NMDA receptors as well as nACh receptors, because both receptors are down-regulated in the brain of Alzheimer's disease patients.

NMDA receptors are known to be modulated by a variety of factors such as polyamines and Mg^{2+} ions. Mg^{2+} ions block the receptors in a voltage-dependent manner, with membrane hyperpolarization intensifying the block (Mayer et al., 1984; Nowak et al., 1984). NMDA receptors are also modulated by protein kinase A (PKA) and protein kinase C (PKC) (Leonard and Hell, 1997), and Mg^{2+} block can be regulated by NMDA and glycine (Liu et al., 2001) and PKC activity (Chen and Huang, 1992). In the present study, we report that Mg^{2+} and PKC play a crucial role in the nefiracetam potentiation of NMDA receptor activity.

Materials and Methods

Cell Preparations. Rat cortical or hippocampal neurons were isolated and cultured by a procedure slightly modified from that described previously (Marszalec and Narahashi, 1993). In brief, rat embryos were removed from 17-day pregnant Sprague-Dawley rats under halothane anesthesia. Small wedges of frontal cortex were excised and incubated in phosphate buffer solution for 20 min at 37°C. This solution contained 154 mM NaCl, 1.05 mM KH_2PO_4 , 3.0 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, and 0.25% (w/v) trypsin (type XI; Sigma-Aldrich, St. Louis, MO), pH 7.4, and osmolality was 287 mOsm. The digested tissue was then mechanically triturated by repeated passages through a Pasteur pipette, and the dissociated cells were suspended in neurobasal medium with B-27 supplement (Invitrogen, Carlsbad, CA) and 2 mM glutamine. The cells were added to 35-mm culture wells at a concentration of 100,000 cells/ml. Each well contained five 12-mm poly-L-lysine-coated coverslips overlaid with confluent glia that had been plated 2 to 4 weeks earlier. The cortical neuron/glia cultures were maintained in a humidified atmosphere of 90% air and 10% CO_2 at 34°C. Cells cultured for 3 to 7 weeks were used for experiments.

Cortical neurons in primary culture were made up of at least three types of neurons: pyramidal neurons, multipolar neurons, and bipolar neurons. Although these three types of neurons generated currents in response to NMDA application, only NMDA currents from pyramidal neurons and multipolar neurons were potentiated by nefiracetam; NMDA currents from bipolar neurons were not affected by nefiracetam. Bipolar neurons were not used in the present study (Moriguchi et al., 2003).

Solutions for Current Recording. The external solution for whole-cell recording of glutamate-induced currents contained 150 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 5.5 mM HEPES acid, 4.5 mM HEPES sodium, and 10 mM D-glucose with various concentrations of MgCl_2 . Tetrodotoxin (100 nM) was added to eliminate the voltage-gated sodium channel currents, and 20 nM atropine sulfate was added to block the muscarinic AChR currents. The pH was 7.3, and the osmolality was adjusted to 300 mOsm with D-glucose. No glycine was added unless otherwise noted. The internal pipette solution

contained 140 mM potassium-gluconate, 2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM HEPES acid, 10 mM EGTA, 2 mM ATP- Mg^{2+} , and 0.2 mM GTP- Na^+ . The pH was adjusted to 7.3 with KOH, and the osmolality was adjusted to 300 mOsm by adding D-glucose.

Whole-Cell Current Recording. Ionic currents were recorded using the whole-cell patch-clamp technique at room temperature (21–22°C). Pipette electrodes were made from 1.5-mm (outer diameter) borosilicate glass capillary tubes with a resistance of 2 to 3 M Ω when filled with the internal solution. The membrane potential was clamped at –70 mV. To study the current-voltage (I-V) relationship for the NMDA currents, NMDA-induced currents were measured while holding the membrane potential at various levels or by using ramp-voltage clamp. For the latter, the membrane potential was changed from –100 to +20 mV in 2 s, and the current-voltage curve was plotted. We allowed 5 to 10 min after membrane rupture for the cell interior to adequately equilibrate with the pipette solution. Currents through the electrode were recorded with an Axopatch-1C amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz, and sampled at 10 kHz in a PC-based data acquisition system that also provided preliminary data analysis. Results are expressed as mean \pm S.E.M., and *n* represents the number of the cells examined.

Drug Applications. Two methods for drug application were used. One method was application via a U-shaped tube (Marszalec and Narahashi, 1993), and the other method was perfusion through the bath. The fast U-shaped tube application system was controlled by a computer-operated magnetic valve system. The valve was normally open to allow the drug solution to bypass the chamber. When it was closed, the drug solution was ejected through the hole of the U-shaped tube to perfuse the cell. At the same time, another valve controlling the suction tube was opened, allowing the test solution to be sucked away quickly. As a result, the external solution surrounding the cell could be completely changed with the drug solution within 30 to 40 ms. Test drugs were also added to the external solution and continuously perfused to the recording chamber via a perfusion system made of glass syringes and Teflon tubings.

Immunoblotting Analysis. Cultured rat cortical neurons were stored in a test tube and kept at –80°C. Neurons were homogenized in 100 μl of homogenizing buffer solution containing 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM Na_3VO_4 , 40 mM sodium pyrophosphate, 50 mM NaF, 100 nM calyculin A, 50 $\mu\text{g}/\text{ml}$ leupeptin, 25 $\mu\text{g}/\text{ml}$ pepstatin A, 50 $\mu\text{g}/\text{ml}$ trypsin inhibitor, and 1 mM dithiothreitol. Insoluble material was removed by a 10-min centrifugation at 15,000 rpm. After determining protein concentration in supernatants using Bradford's solution, samples were boiled 3 min in Laemmli sample buffer (Laemmli, 1970). Samples containing the equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to an Immobilon polyvinylidene difluoride membrane for 2 h at 70 V. After blocking with (50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.5, containing 2.5% bovine serum albumin for 1 h at room temperature, membranes were incubated overnight at 4°C with anti-phospho-PKC (pPKC) (1:2000; Upstate Biotechnology, Lake Placid, NY), anti-phospho-MARCKS (Ser-152/156) (1:2000; Chemicon International, Temecula, CA), anti-MARCKS (1:2000; Ohmitsu et al., 1999), anti-phospho-NR1 (Ser-896) (1:2000; Upstate Biotechnology), anti-NR1 (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phospho-GluR1 (Ser-845) (1:1000; Upstate Biotechnology), anti-GluR1 (1:1000; Chemicon International), or anti-phospho-dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) (Thr-34) (1:2000; Cell Signaling Technology Inc., Danvers, MA). Bound antibodies were visualized using the enhanced chemiluminescence detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and analyzed semiquantitatively using the NIH Image software (<http://rsb.info.nih.gov/nih-image/>).

Chemicals. NMDA (Sigma-Aldrich) and glycine (Sigma/RBI, Natick, MA) were first dissolved in distilled water to make stock solutions. The muscarinic AChR blocker atropine sulfate, the PKC inhibitor chelerythrine chloride, the PKA activator 8-bromo-cAMP,

and the PKC activator phorbol 12-myristate 13-acetate were purchased from Sigma-Aldrich. The PKA inhibitor H89 was obtained from Calbiochem-Novabiochem (San Diego, CA). Nefiracetam [DM-9384; *N*-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide] was provided by Daiichi Pharmaceutical Co. (Tokyo, Japan), and it was first dissolved in distilled water to make stock solutions. The stock solutions of nefiracetam were stored at 4°C and diluted to prepare test solutions with the external solution shortly before the experiments.

Data Analysis. Current records were initially analyzed by the Clamp-Fit module of the PClamp6 program (Molecular Devices) to assess whole-cell current amplitude and decay kinetics. Data are expressed as the mean \pm S.E.M. unless otherwise stated. The concentration-response data were subsequently compiled for graphical analysis in SigmaPlot 8.0 (SPSS Inc., Chicago, IL). Analysis of variance and Student's *t* tests were performed to assess significance of differences, if applicable. *p* values less than 0.05 are considered statistically significant.

Results

PKC Inhibition Reverses Nefiracetam Potentiation, but PKA Inhibition Does Not. The activity of NMDA receptors is known to be modulated by protein kinases (Ben-Ari et al., 1992; Chen and Huang, 1992; Leonard and Hell, 1997; Logan et al., 1999). To see whether PKA or PKC was involved in the nefiracetam potentiating action on NMDA receptors, specific inhibitors of these kinases were used in the absence of Mg^{2+} in the external solutions.

Chelerythrine is a membrane-permeable-specific inhibitor of PKC, and H89 is a membrane-permeable PKA inhibitor. Nefiracetam at 10 nM greatly increased the current evoked by 30 μ M NMDA to 160 to 180% of the control (Fig. 1). When chelerythrine at 3 μ M was applied during the current potentiation caused by 10 nM nefiracetam, a complete block of the nefiracetam effect was observed (Fig. 1). However, H89 applied at 1 μ M while the current was being potentiated by 10 nM nefiracetam failed to abolish nefiracetam potentiation (Fig. 1).

Bath perfusion of 3 μ M chelerythrine slightly suppressed the currents induced by 30 μ M NMDA to $82.1 \pm 7.2\%$ of the control ($n = 4$; $p < 0.05$) (Fig. 2). Addition of 10 nM nefiracetam to the bathing solution no longer produced current potentiation. Washing with nefiracetam- and chelerythrine-free solution tended to restore the current, but the recovery reached only $79.6 \pm 8.7\%$ of the control after 10 min of washing (Fig. 2). By contrast, the PKA inhibitor H89 did not prevent nefiracetam potentiation of NMDA-induced currents (Fig. 2). H89 by itself at 1 μ M slightly but insignificantly suppressed the currents to $90.8 \pm 4.7\%$ of the control ($n = 4$; $p > 0.05$). Addition of 10 nM nefiracetam caused a robust increase in the currents to $157.4 \pm 4.2\%$ of the control ($n = 4$). Washing for 10 min with nefiracetam- and H89-free solutions restored the currents to $86.6 \pm 4.6\%$ of the control. Thus, it was concluded that nefiracetam potentiation of NMDA currents required active PKC but not PKA. The observation that both PKC and PKA inhibitors cause a small reduction in the NMDA current (Fig. 2) suggests that there are some tonic activation of PKA and PKC. The incomplete recovery after washing out the inhibitors and nefiracetam might reflect a rundown in the NMDA current.

Nefiracetam Enhances PKC α Autophosphorylation and PKC Phosphorylation of MARCKS and NR1. PKC α is a major PKC isoform expressed in the hippocampus (Sieber

et al., 1998). Because nefiracetam potentiation of NMDA currents is regulated by PKC, we hypothesized that nefiracetam might modulate PKC α autophosphorylation. Nefiracetam treatment potentiated PKC α autophosphorylation in a manner following a bell-shaped dose-response relationship that peaked at 10 nM ($138.1 \pm 7.0\%$ of control; $p < 0.05$; $n = 6$) (Fig. 3). We also assessed phosphorylation of MARCKS (Ohmitsu et al., 1999) and NR1 (Tingley et al., 1997) as downstream PKC α targets using phospho-specific antibodies. Consistent with the increase in PKC α autophosphorylation, nefiracetam treatment at 10 nM significantly increased MARCKS ($146.0 \pm 7.8\%$ of the control; $p < 0.05$; $n = 6$) and NR1 phosphorylation ($143.4 \pm 7.3\%$ of control; $p < 0.05$; $n = 6$) (Fig. 3B) without changing their protein levels (Fig. 3A). The bell-shaped increases in PKC α autophosphorylation and phosphorylation of its substrates are in accord with the bell-shaped potentiation of NMDA receptor currents caused by nefiracetam as observed previously (Moriguchi et al., 2003).

The nefiracetam-induced increase in phosphorylation was prevented by PKC inhibitors as illustrated in Fig. 4. Again,

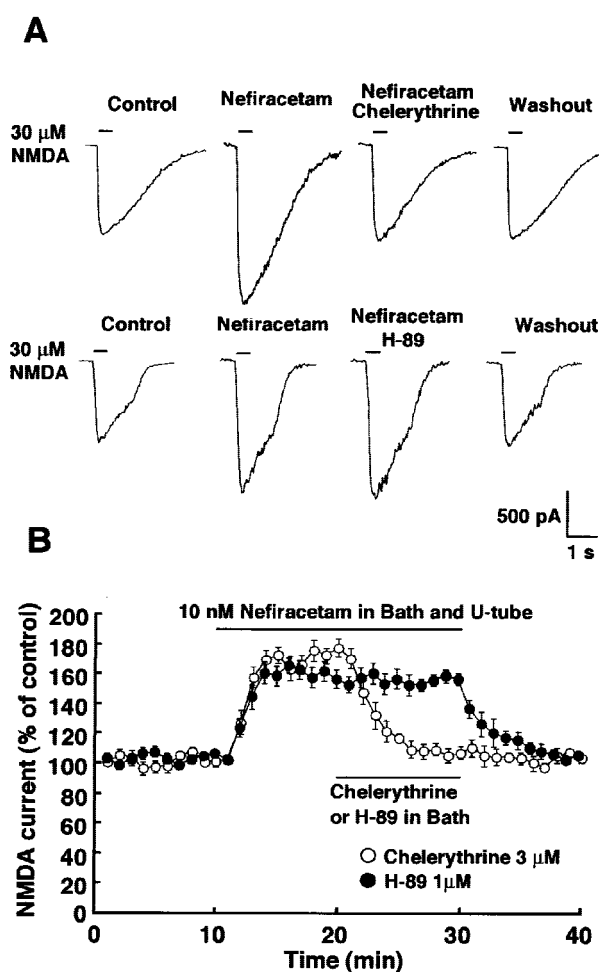


Fig. 1. The PKC inhibitor chelerythrine reverses the potentiation of the NMDA currents by nefiracetam, but the PKA inhibitor H89 does not. Currents were evoked at a holding potential of -70 mV by 30 μ M NMDA at a 1-min interval. No glycine and Mg^{2+} were added to the external solutions. A, currents recorded before, during treatment with 10 nM nefiracetam, during treatment with 10 nM nefiracetam and 3 μ M chelerythrine or 1 μ M H89, and after washout of nefiracetam and chelerythrine or H89. B, time course of changes in the peak current amplitude as shown in A (mean \pm S.E.M.; $n = 4$).

10 nM nefiracetam treatment increased autophosphorylated PKC to $142.6 \pm 2.5\%$ of the control ($p < 0.05$; $n = 6$) and pNR1 to $130.8 \pm 10.2\%$ of the control ($p < 0.05$; $n = 6$). These increases in phosphorylated peptides pPKC α and pNR1 were prevented by PKC inhibitor chelerythrine. The total level of PKC α and the NR1 proteins was not changed by either nefiracetam or nefiracetam and chelerythrine (Fig. 4).

To verify the specificity of the increase in pPKC activity by nefiracetam treatment, we also investigated phosphorylation of GluR1 (Ser-845) and the dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) (Thr-34) as the downstream targets of PKA (Roche et al., 1996; Edwards et al., 2002). Nefiracetam treatment did not affect phosphorylation of GluR1 (Ser-845) and DARPP-32 (Thr-34) (Fig. 5).

Nefiracetam Reduces Mg^{2+} Block of NMDA Currents: Role of PKC. The NMDA receptor activity is known to be modulated by external Mg^{2+} ions. Because the dose-response relationship for nefiracetam potentiation follows a bell-shaped curve, with the potentiation reaching a maximum at 10 nM and decreasing at higher concentrations (Moriguchi et al., 2003), 10 nM nefiracetam was chosen to study Mg^{2+} block of the NMDA receptor. NMDA was applied via a U-shaped tube at 30 μ M, and Mg^{2+} at various concen-

trations was applied via both U-shaped tube and bathing solution.

At a holding potential of -70 mV, Mg^{2+} suppressed currents induced by NMDA in a dose-dependent manner (Fig. 6, A and C). Mg^{2+} suppression was observed at 10 μ M, and near complete block occurred at 1000 μ M. When 10 nM nefiracetam was applied via a U-shaped tube and bath, NMDA-induced currents were greatly potentiated in the absence of Mg^{2+} in the bath (Fig. 6, B and C). Addition of Mg^{2+} at concentrations of 1 to 100 μ M slightly suppressed the currents, but substantial currents remained even at the maximum concentration of 1000 μ M. Dose-response relationships for Mg^{2+} suppression of NMDA currents without (\circ) and with (\bullet) nefiracetam are shown in Fig. 6C. This shows that nefiracetam reduces Mg^{2+} block to a greater extent as the Mg^{2+} concentration is increased.

The experiments described above showed that there were interactions between nefiracetam and Mg^{2+} for modulation of NMDA-induced currents. To see the interactions between Mg^{2+} and nefiracetam more clearly, I-V relationships were obtained by the ramp-voltage clamp technique in the pres-

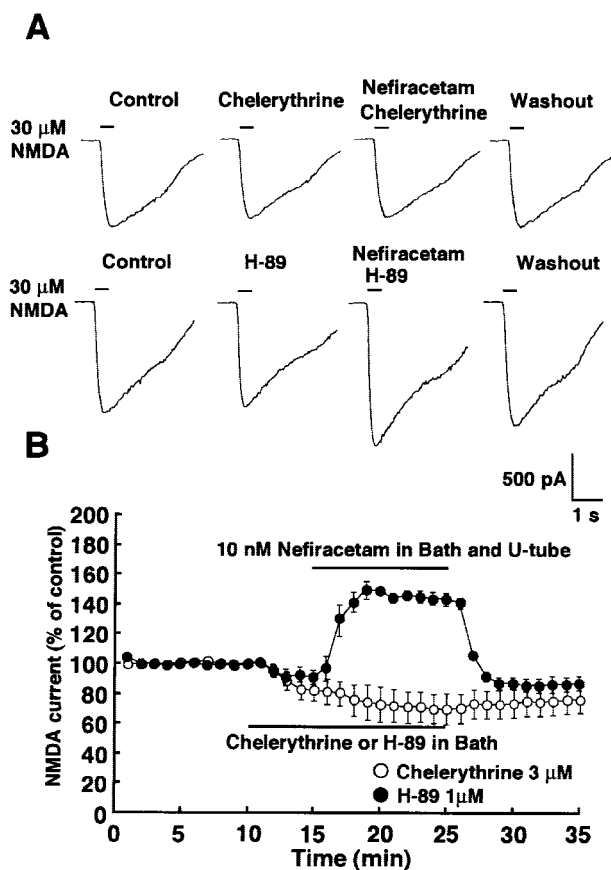


Fig. 2. The PKC inhibitor chelerythrine prevents nefiracetam potentiation of NMDA currents, but the PKA inhibitor H89 does not. Currents were evoked at a holding potential of -70 mV by 30 μ M NMDA at a 1-min interval. No glycine and Mg^{2+} were added to the external solutions. A, currents recorded before treatment, during treatment with 3 μ M chelerythrine or 1 μ M H89, during treatment with 3 μ M chelerythrine or 1 μ M H89 plus 10 nM nefiracetam, and after washout of nefiracetam and chelerythrine or H89. B, time course of changes in the peak current amplitude as shown in A (mean \pm S.E.M.; $n = 4$).

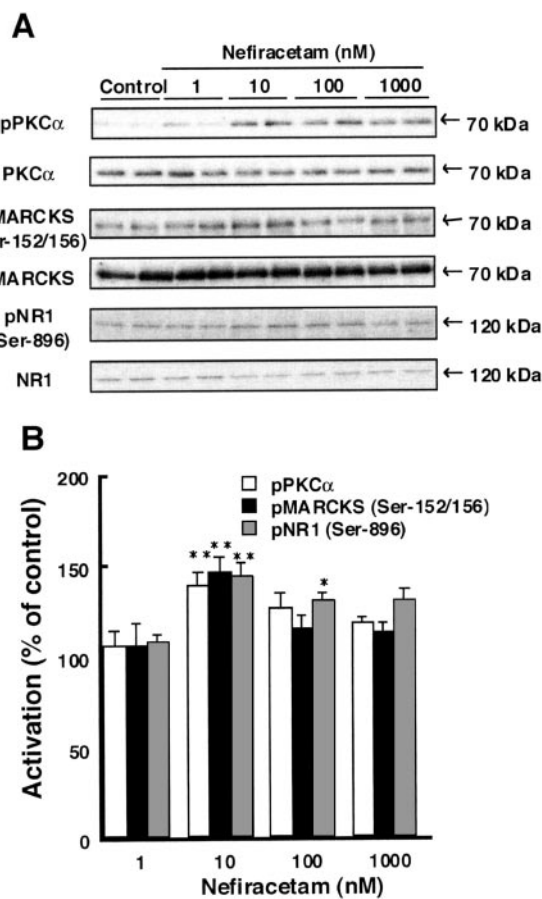


Fig. 3. Effects of nefiracetam treatment on PKC α autophosphorylation and phosphorylation of MARCKS and NR1 (Ser-896). A, representative images of immunoblots using antibodies against pPKC α , PKC α , phosphorylated MARCKS (Ser-152/156), MARCKS, phosphorylated pNR1 (Ser-896), and NR1. Protein levels were unchanged after nefiracetam treatment. B, quantitative measurements of pPKC α , pMARCKS (Ser-152/156), and pNR1 (Ser-896) analyzed by densitometry. Data are expressed as percentages of the controls without stimulation. *, $p < 0.05$; **, $p < 0.01$ versus the control. $n = 6$. A bell-shaped dose-response relationship showed that nefiracetam maximally phosphorylated PKC α , MARCKS, and NR1 at 10 nM.

ence of 3 μM glycine (Fig. 7). The voltage-dependent block caused by 1 mM Mg^{2+} was observed at negative membrane potentials. The voltage-dependent block by 1 mM Mg^{2+} was greatly reduced by 10 nM nefiracetam, changing from the inward-rectifying I-V relationship to a near linear relationship. Thus, at -70 mV, the current amplitude was increased more than 4-fold by 10 nM nefiracetam.

Magnesium block of NMDA currents was evaluated by extrapolating the linear portion of I-V curve to -70 mV. Mg^{2+} at 1 mM reduced the current to $15.3 \pm 5.2\%$ ($n = 5$) of the extrapolated value. The current amplitude in 10 nM nefiracetam was $78.3 \pm 8.5\%$ ($n = 4$) of the extrapolated value. Addition of 3 μM chelerythrine to nefiracetam decreased the current to $22.4 \pm 6.5\%$ ($n = 4$). Thus, it was concluded that nefiracetam reduced the Mg^{2+} -induced voltage-dependent block of NMDA currents, which was sensitive to the PKC inhibitor chelerythrine.

Effects of Glycine and Mg^{2+} on NMDA Current-Voltage Relationship. In the previous study (Moriguchi et al., 2003), we took a simplest interpretation that nefiracetam interacted with the glycine site of NMDA receptors. The present study showed that nefiracetam attenuated voltage-dependent Mg^{2+} block (Fig. 7). These results raised a question as to whether nefiracetam works on both PKC and glycine sites. To examine this possibility, we tested whether nefiracetam acts similarly to glycine to increase the NMDA current. Ramp-voltage clamp experiments were performed to plot current-voltage relationships for the currents induced by 100 μM NMDA at low (100 nM) and high (3 μM) glycine

concentrations and in the presence and absence of 1 mM Mg^{2+} using a hippocampal neuron (Fig. 8A). Traces 1 and 2 were obtained, respectively, in 100 nM glycine plus 1 mM Mg^{2+} and 100 nM glycine without Mg^{2+} . Voltage-dependent Mg^{2+} block occurred in the presence of 100 nM glycine. Traces 3 and 4 were obtained, respectively, in 3 μM glycine plus 1 mM Mg^{2+} and 3 μM glycine without Mg^{2+} . Again, voltage-dependent Mg^{2+} block was observed in the presence of high (3 μM) glycine concentration. Experiments with step voltage changes yielded similar results. Figure 8B shows an example of an experiment in 3 μM glycine and 1 mM Mg^{2+} . Voltage-dependent Mg^{2+} block is clearly seen in the presence of a high concentration of Mg^{2+} . Thus, the current-voltage relationship remained inwardly rectifying when the NMDA current was increased by high concentrations of glycine. It seems that glycine and nefiracetam act differently to modulate the Mg^{2+} site on the NMDA receptor.

Discussion

The present study extended our previous article (Moriguchi et al., 2003) to determine the roles of second messengers and Mg^{2+} in nefiracetam-induced potentiation of NMDA receptor activity. Our previous study showed that nefiracetam interacted with the glycine binding site on the NMDA receptors, thereby potentiating the NMDA-induced current. It has now been shown that nefiracetam potentiation of NMDA currents was not affected by the inhibition of PKA but abol-

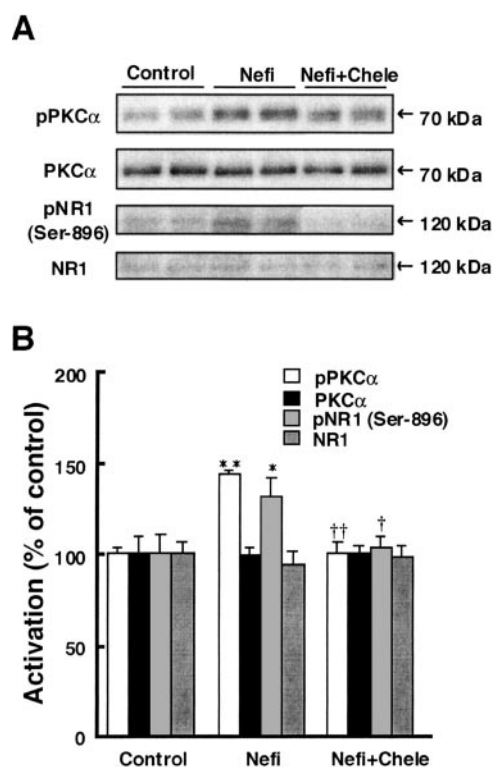


Fig. 4. PKC inhibitor chelerythrine prevents phosphorylation of PKC α and NR1 subunit. The phosphorylated fraction of PKC α (pPKC α) and that of the NR1 subunit (pNR1) were increased by 10 nM nefiracetam to 142.6 ± 2.5 and $130.8 \pm 10.2\%$ of their control levels, respectively ($p < 0.05$; $n = 6$). Chelerythrine treatment prevented such increases. The total protein levels of PKC α and NR1 subunits were not changed by nefiracetam.

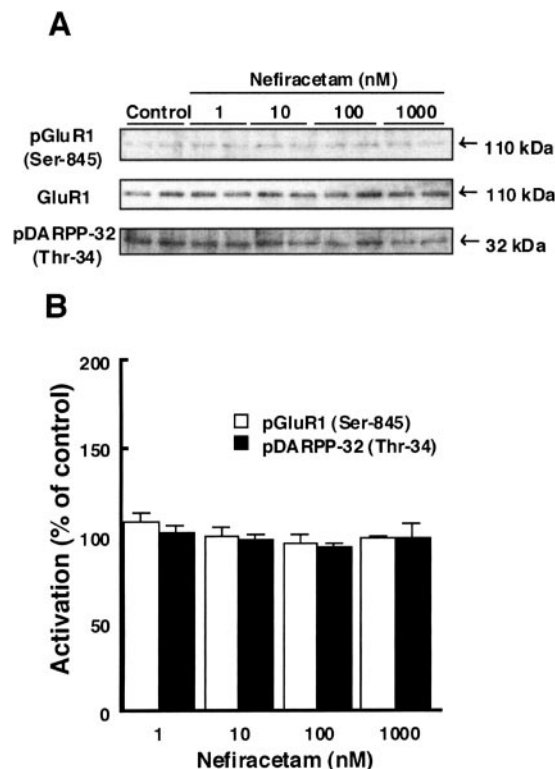


Fig. 5. Nefiracetam treatment does not affect phosphorylation of GluR1 (Ser-845) and DARPP-32. A, representative images of immunoblots using antibodies against phosphorylated pGluR1 (Ser-845), GluR1, and phosphorylated DARPP-32 (Thr-34). GluR1 protein levels were unchanged after treatment. B, quantitative analyses of phosphorylated pGluR1 (Ser-845) and phosphorylated DARPP-32 (Thr-34) determined by densitometry. Data are expressed as percentages of the controls without stimulation. $n = 6$.

ished when PKC was inhibited. Thus, PKC plays a crucial role in nefiracetam potentiation of NMDA-induced currents by phosphorylating the NR1 subunit as seen with the activation of group II metabotropic glutamate receptors (Tyszkiewicz et al., 2004). Nefiracetam also decreased the degree of voltage-dependent Mg^{2+} block of NMDA currents. These results are consistent with the previous observation (Chen and Huang, 1992) that Mg^{2+} block is reduced by PKC activation.

NMDA receptors are formed by the obligatory NR1 subunits in various combinations with NR2A-NR2D subunits (Kutsuwada et al., 1992; Monyer et al., 1992), despite the fact that a functional receptor can be formed with the NR1 subunit alone. The NMDA receptor activity is highly sensitive to the state of phosphorylation, because the NR1, NR2A, and NR2B subunits are substrates for PKA and PKC (Tingley et al., 1993; Leonard and Hell, 1997; Swope et al., 1999). Phosphorylation of these subunits at different sites produces an additive potentiating effect on the NMDA receptor activity. Phosphorylation of the NR1 subunit in the hippocampus was enhanced by the activators of PKC, but not by those of PKA, and the enhancement by PKC was inhibited by PKC inhibitors (Suen et al., 1998). A similar result was obtained with the prefrontal cortex by the activation of group II metabotropic glutamate receptors (Tyszkiewicz et al., 2004).

A bell-shaped increase in PKC α autophosphorylation and concomitant increases in phosphorylation of MARCKS and NR1 (Ser-896) (Fig. 3) resembled the potentiation of NMDA currents induced by nefiracetam (Moriguchi et al., 2003). The PKC isoform was found to be associated with the NR1 subunit, and increased NR1 phosphorylation by PKC is particularly important for long-term potentiation, because NR1 phosphorylation accounts for up-regulation of NMDA receptor function (Tingley et al., 1993). In addition, two mechanisms have been proposed to increase the NMDA currents. One of the mechanisms for potentiation of the NMDA response by PKC is to phosphorylate the NR1 subunit, thereby reducing its affinity for calmodulin and resulting in a reduction in its ability to inactivate the NMDA receptor in a Ca^{2+} -dependent manner (Hisatsune et al., 1997). Another possibility is the removal of Mg^{2+} block without depolarization (Chen and Huang, 1992; Pittaluga et al., 2000). These

results suggest that the PKC pathway plays a major role in the potentiating action of nefiracetam on the NMDA receptors in multipolar neurons in primary culture. It remains to be determined whether PKC activation increases tyrosine phosphorylation of NR2 subunits via activation of the Src kinases (Salter and Kalia, 2004).

The inhibition of PKA (Figs. 1 and 2) exerted little or no effect on the NMDA receptor activity despite the fact that both the NR1 and NR2 subunits are substrates of PKA. Likewise, forskolin, an activator of adenylate cyclase, had no effect on NMDA currents in cultured hippocampal pyramidal neurons (Greengard et al., 1991). In contrast, stimulation of PKA enhanced NMDA responses in the neostriatal neurons (Colwell and Levine, 1995) and in the rat spinal dorsal horn neurons (Cerne et al., 1993), where phosphorylation of the NR1 subunit was reduced by H89, a PKA inhibitor (Zou et al., 2002). It seems that modulation of NMDA responses by PKA is specific for brain regions.

It is also possible that PKA modulation of the NMDA receptor is more labile than PKC. PKA activation enhanced the NMDA receptor response in the hypothalamic neurons, but not in the recombinant NMDA receptors when the NR1, NR2A, and/or NR2B subunits were expressed in *Xenopus laevis* oocytes (Nijholt et al., 2000). However, PKA modulation of the NMDA activity was observed when rat striatal poly(A)⁺ mRNA was injected in oocytes (Blank et al., 1997), suggesting that an additional protein component was required for PKA modulation of the NMDA receptors. Inhibition of protein phosphatase 1 and/or protein phosphatase 2A by the specific inhibitor calyculin A occluded the PKA-mediated potentiation of striatal NMDA responses, suggesting that the PKA effect was mediated by inhibition of a protein phosphatase (Blank et al., 1997; Nijholt et al., 2000). DARPP-32, an endogenous phosphatase inhibitor, which is enriched in the striatum, might participate in the PKA regulation of the NMDA currents in these neurons (Blank et al., 1997). Figure 5 showing the lack of effects of nefiracetam on pDARPP-32 is consistent with the other results that the cAMP-PKA pathway is not involved in the potentiating action of nefiracetam. In addition, the observation that nefiracetam did not alter phosphorylated states of GluR1 is consis-

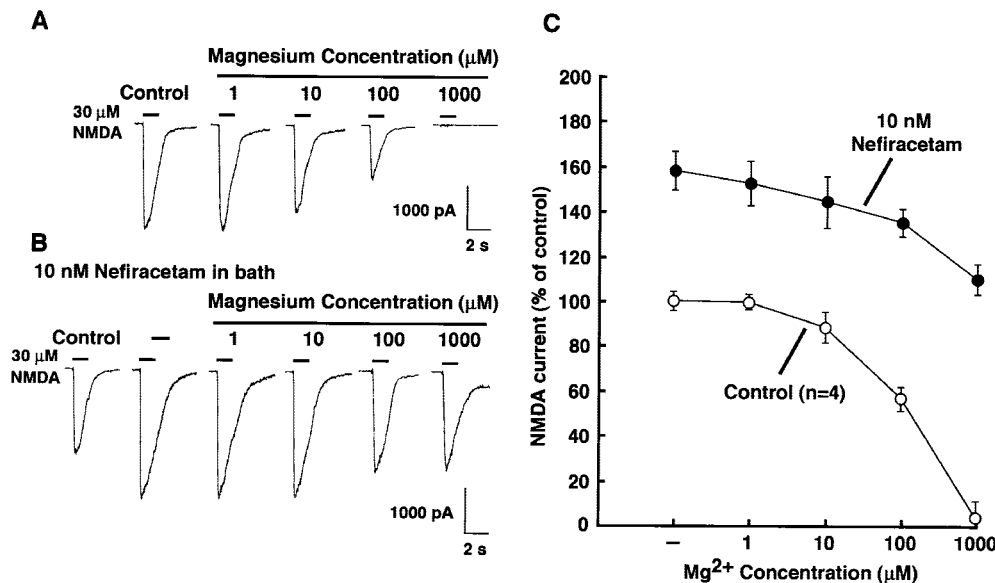


Fig. 6. Nefiracetam reduces Mg^{2+} block of NMDA currents in cortical neurons. Currents were evoked at a holding potential of -70 mV by 250-ms pulses of $30 \mu M$ NMDA via a U-shaped tube system at an interval of 1-min. Mg^{2+} was applied to the bath at various concentrations. A, Mg^{2+} suppressed NMDA currents in a dose-dependent manner. B, nefiracetam at 10 nM potentiated NMDA currents and reduced Mg^{2+} block. C, dose-dependent suppression of NMDA currents caused by Mg^{2+} (mean \pm S.E.M.; $n = 4$). Current amplitude is plotted as the percentage of the control current amplitude without Mg^{2+} and nefiracetam. Without nefiracetam (control, \circ), Mg^{2+} suppressed the current dose-dependently. When 10 nM nefiracetam was present in the bath, Mg^{2+} suppression was greatly reduced (\bullet).

tent with our previous result that nefiracetam has no effect on α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors.

The voltage-dependent Mg^{2+} block of NMDA currents was greatly attenuated by nefiracetam in a concentration-dependent manner. Because Mg^{2+} blocks the NMDA receptor at the resting membrane potential in normal physiological conditions, attenuation of Mg^{2+} block caused by nefiracetam represents one of the important mechanisms whereby nefiracetam would potentiate the activity of NMDA receptors. Consistent with this notion is that the voltage dependence of

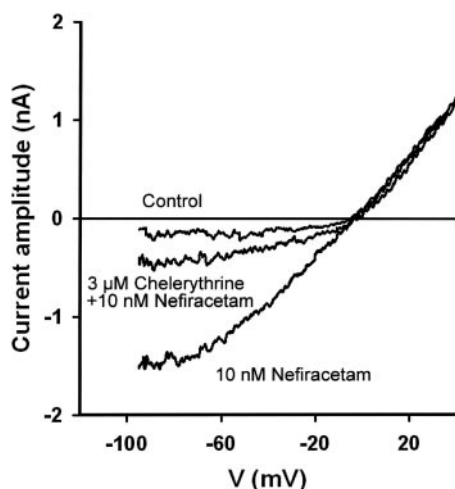


Fig. 7. Nefiracetam reduces voltage-dependent Mg^{2+} block of NMDA current in a cortical neuron. NMDA responses were induced by the U-shaped tube applications of 100 μ M NMDA and 3 μ M glycine, and the current-voltage relationships were obtained by the ramp-voltage clamp methods (a ramp changing from -100 to $+40$ mV in 2 s). Nefiracetam 10 nM was applied to the bath, and Mg^{2+} was present in the external solution at a concentration of 1 mM. In the control measurement without nefiracetam, Mg^{2+} suppression was seen at negative membrane potentials. When nefiracetam was applied to the bath, Mg^{2+} suppression of NMDA currents was greatly attenuated. After 5 min of treatment with 3 μ M chelerythrine, the effect of nefiracetam to remove Mg^{2+} block was largely lost.

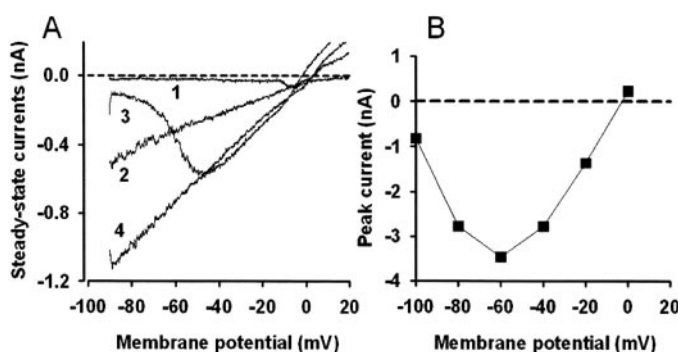


Fig. 8. Current-voltage relationship for NMDA currents in different concentrations of glycine and magnesium ions. **A**, current-voltage relationships in a hippocampal neuron obtained by the ramp-voltage clamp method during the steady-state current at potentials ranging from -90 mV to $+20$ mV in 2 s. Trace 1 was recorded in 100 nM glycine and 1 mM Mg^{2+} , and trace 2 was recorded in 100 nM glycine and without 1 mM Mg^{2+} . Trace 3 was recorded in 3 μ M glycine and 1 mM Mg^{2+} , and trace 4 was recorded in 3 μ M glycine and no added Mg^{2+} . The NMDA concentration used was 100 μ M in all cases. The voltage-dependent block by Mg^{2+} is most clearly seen in both traces 1 and 3. **B**, current-voltage relationship for NMDA currents obtained from a cortical neuron by step voltage changes. The peak currents induced by 100 μ M NMDA were measured as a function of holding membrane potentials in 3 μ M glycine and 1 mM Mg^{2+} .

Mg^{2+} block of the NMDA receptor was attenuated by nefiracetam.

In a previous article, we showed that nefiracetam acted as a partial agonist at the glycine binding site of the NMDA receptor (Moriguchi et al., 2003). There are several observations indicating that the increase in the NMDA current by increasing glycine concentrations differs from the increase caused by nefiracetam. First, glycine exerts its coagonistic action rapidly, whereas nefiracetam takes several minutes to act. Second, the current-voltage relationship for the NMDA receptor measured in 1 mM Mg^{2+} ions is inwardly rectifying in the presence of 3 μ M glycine (Fig. 8), whereas the I-V relationship is almost linearized by nefiracetam (Fig. 7). The modulatory effects of nefiracetam on the glycine site and Mg^{2+} site are both sensitive to the PKC inhibitor chelerythrine. These observations have led us to reinterpret that nefiracetam acts indirectly to enhance the affinity of glycine for the NR1 subunit by phosphorylation via activation of PKC. The removal of Mg^{2+} block by nefiracetam might involve an intersubunit interaction, because the N site and N + 1 site on the NR2 subunit are involved in Mg^{2+} block (Wollmuth et al., 1998), and also because nefiracetam phosphorylates the NR1 subunit. If the NR2 subunit can be tyrosine phosphorylated by Src kinases via PKC activation, then the effect on Mg^{2+} block might represent intrasubunit modulation.

The effect of nefiracetam on the glycine site was previously examined in a nominal Mg^{2+} -free condition (Moriguchi et al., 2003). In the present study, the removal of Mg^{2+} block by nefiracetam was examined in the presence of a high glycine concentration (Fig. 7). In the absence of nefiracetam, glycine and Mg^{2+} do not seem to interact to modulate NMDA receptor activity (Fig. 8). However, glycine at higher concentrations has been found to modulate Mg^{2+} block depending on pH and Ca^{2+} ion concentrations (Liu et al., 2001). It remains to be determined in the presence of nefiracetam whether glycine and Mg^{2+} interact to modulate the NMDA receptor activity.

The effect of PKC in nefiracetam action on various sites needs further clarification, because there has been some controversy regarding its role. In our previous study, inhibition of PKC had no immediate effect on nefiracetam-induced potentiation of $\alpha 4\beta 2$ -type nACh receptors in rat cortical neurons, and the nefiracetam potentiation of acetylcholine currents occurred via G_s proteins (Zhao et al., 2001). However, PKC was reported to play an important role in the nefiracetam potentiation of the activity of the $\alpha 4\beta 2$ and $\alpha 7$ nACh receptors expressed in *Xenopus laevis* oocytes (Nishizaki et al., 2000a,b), and in CA1 neurons in the hippocampus (Nishizaki et al., 1999). Nefiracetam failed to potentiate the $\alpha 4\beta 2$ nicotinic acetylcholine receptors when they were expressed in human embryonic kidney cells (Zhao et al., 2001). It was suggested that such controversy may be due to different PKC isoforms involved in different tissues (Nishizaki et al., 2000b).

Because there is down-regulation of both nACh receptors and NMDA receptors in the brain of Alzheimer's disease patients, potentiation of both receptors by nefiracetam (Zhao et al., 2001; Moriguchi et al., 2003) may improve patient learning and memory. Alternatively, overstimulation of NMDA receptors causes toxicity and cell death. The role of NMDA receptor in disease states forms a continuum from pathologically low to high levels (Hardingham and Bading,

2003). Thus, restoring the synaptic NMDA receptor activity to the normal level may be crucial for the therapeutic effects of nefiracetam.

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