Potentiation of \( N \)-Methyl-\( D \)-aspartate-Induced Currents by the Nootropic Drug Nefiracetam in Rat Cortical Neurons

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ABSTRACT

Nefiracetam is a new pyrrolidone nootropic drug being developed for the treatment of Alzheimer’s type and post-stroke vascular-type dementia. In the brain of Alzheimer’s disease patients, down-regulation of both cholinergic and glutamatergic systems has been found and is thought to play an important role in impairment of cognition, learning, and memory. We have previously shown that the activity of neuronal nicotinic acetylcholine receptors is potently augmented by nefiracetam. The present study was undertaken to elucidate the mechanism of action of nefiracetam on glutamatergic receptors. Currents were recorded from rat cortical neurons in long-term primary culture using the whole-cell patch-clamp technique at a holding potential of −70 mV in Mg\(^2+\)-free solutions. \( N \)-Methyl-\( D \)-aspartate (NMDA)-evoked currents were greatly and reversibly potentiated by bath application of nefiracetam resulting in a bell-shaped dose-response curve. The minimum effective nefiracetam concentration was 1 nM, and the maximum potentiation to 170% of the control was produced at 10 nM. Nefiracetam potentiation occurred at high NMDA concentrations that evoked the saturated response, and in a manner independent of NMDA concentrations ranging from 3 to 1,000 \( \mu \)M. Glycine at 3 \( \mu \)M potentiated NMDA currents but this effect was attenuated with an increasing concentration of nefiracetam from 1 to 10,000 nM. 7-Chlorokynurenic acid at 1 \( \mu \)M prevented nefiracetam from potentiating NMDA currents. Nefiracetam at 10 nM shifted the dose-response relationship for the 7-chlorokynurenate inhibition of NMDA currents in the direction of higher concentrations. \( \alpha \)-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid- and kainate-induced currents were not significantly affected by application of 10 nM nefiracetam. It was concluded that nefiracetam potentiated NMDA currents through interactions with the glycine binding site of the NMDA receptor.

It is well established that accumulation of \( \beta \)-amyloid in the brain is a hallmark of Alzheimer’s disease. However, no strategies for curing the disease have been developed yet, because we do not know the exact cause of the disease (Hirai, 2000). It is also 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. This approach has indeed proven successful to a limited extent, and the U.S. Food and Drug Administration has approved four anticholinesterases, tacrine, donepezil, rivastigmine, and galantamine, for the treatment of Alzheimer’s disease patients. These drugs, being anticholinesterases, increase the amount of acetylcholine (ACh) in the synaptic cleft thereby stimulating the cholinergic system, but they cause some side effects such as nausea, diarrhea, and vomiting. Furthermore, their efficacy in improving cognition, learning, and memory is somewhat limited.

Under the circumstances, newer approaches are urgently required. One of these approaches is to directly stimulate neuronal nicotinic acetylcholine (\( n \)ACh) receptors in the brain. It has indeed been demonstrated recently that nefiracetam (Zhao et al., 2001) and galantamine (Schrattenholz et al., 1996; Maelike and Albuquerque 2000; Maelicke et al., 2001) potentiate ACh-induced currents in \( n \)ACh receptors. Although galantamine does inhibit cholinesterase, its potency is low. The optimal concentration of galantamine to maximally potentiate ACh-induced currents is 0.1 to 1 \( \mu \)M (Schrattenholz et al., 1996; Maelike and Albuquerque 2000; Maelicke et al., 2001; Santos et al., 2002). Nefiracetam is extremely potent (0.1–1.0 nM), and it efficaciously potentiates ACh-induced currents to 200% of the control in the \( \alpha \)4\( \beta \)2-type ACh receptors in rat cortical neurons (Zhao et al., 2001). Thus, direct potentiation of \( n \)ACh receptor activity is a promising approach.

Alzheimer’s disease is a progressive neurodegenerative disorder of cognitive function. Reductions in \( N \)-methyl-\( D \)-as-

**ABBREVIATIONS:** NMDA, \( N \)-methyl-\( D \)-aspartate; ACh, acetylcholine; \( n \)ACh, nicotinic ACh; AMPA, \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; 7-CIKN, 7-chlorokynurenate acid.
partate (NMDA) receptors are also found in the brain of Alzheimer’s patients, possibly contributing to memory deficits (Greenamyre et al., 1987; Cowburn et al., 1990; Fonnum et al., 1995; Sze et al., 2001). Drugs that modulate NMDA receptor-mediated neural transmission by acting at the glycine binding site of NMDA receptors are potential therapeutic agents to treat memory deficits associated with aging and Alzheimer’s disease. Both the partial glycine site agonist d-cycloserine and the glycine prodrug milacemide prevent memory deficit in animal paradigms (Hanndelmann et al., 1989; Baxter et al., 1994), and have been tested as cognitive enhancers in both healthy subjects and patients with Alzheimer’s disease (Schwartz et al., 1991, 1996; Dysken et al., 1992). Thus, NMDA receptors play a crucial role in learning and memory. Our working hypothesis is that one of the mechanisms by which nootropic drugs improve cognitive function is by increasing the activity of NMDA receptors as well as nACh receptors in the brain of Alzheimer’s disease patients who have down-regulated NMDA receptors. In post-stroke patients who have excess glutamate release, nootropic drugs, being partial agonists, reduce the excess activation of NMDA receptors.

We have found in the present study that nefiracetam potently augments NMDA-evoked currents in rat cortical neurons. This action appears to be exerted via nefiracetam interactions with the glycine binding site on the NMDA receptors.

Materials and Methods

Cell Preparation. Rat cortical neurons were 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 subsequently incubated in the phosphate buffer solution for 20 min at 37°C. This solution contained 154 mM NaCl, 1.05 mM KH2PO4, 3.0 mM Na2HPO4·7H2O, 0.25% (w/v) trypsin (type XI; Sigma-Aldrich, St. Louis, MO), pH 7.4, with an osmolarity of 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 multipolar neuron. NMDA was applied at a concentration of 10 μM to the neuron via a U-tube for 250 ms at an interval of 1 min. Neither Mg2+ nor glycine was added to the external solution. Bath application of 10 nM nefiracetam greatly potentiated the NMDA-induced current, and the effect was completely reversible after washing with nefiracetam-free solution. The time course of changes in NMDA current amplitude during and after bath application of 10 nM nefiracetam is illustrated in Fig. 1B. The current amplitude was increased by nefiracetam to ~1700 pA from the control level of ~1100 pA, representing about a 50% increase. In contrast, smaller bipolar neurons did not respond to 10 nM nefiracetam, with the NMDA current amplitude remaining nearly constant during and after bath perfusion of nefiracetam (Fig. 1, C and D). All of the subsequent experiments were performed using multipolar neurons unless otherwise stated. Figure 1A shows an example of an experiment using a multipolar neuron. NMDA was applied at a concentration of 30 μM to the neuron via a U-tube for 250 ms at an interval of 1 min. Neither Mg2+ nor glycine was added to the external solution. Bath application of 10 nM nefiracetam greatly potentiated the NMDA-induced current, and the effect was completely reversible after washing with nefiracetam-free solution. The time course of changes in NMDA current amplitude during and after bath application of 10 nM nefiracetam is illustrated in Fig. 1B. The current amplitude was increased by nefiracetam to ~1700 pA from the control level of ~1100 pA, representing about a 50% increase. In contrast, smaller bipolar neurons did not respond to 10 nM nefiracetam, with the NMDA current amplitude remaining nearly constant during and after bath perfusion of nefiracetam (Fig. 1, C and D). All of the subsequent experiments were performed using multipolar neurons unless otherwise stated. Dose-response relationships for nefiracetam potentiation of NMDA-induced currents are shown in Fig. 2. The minimal effective concentration of nefiracetam to potentiate NMDA currents in multipolar cells was 1 nM at which only about 10% current potentiation over and above control was observed (Fig. 2A). Nefiracetam at 10 nM greatly potentiated the current by 70%, but further increases in the concentra-
tion to 100 and 1000 nM caused a decrease in efficacy (Fig. 2A). This resulted in a bell-shaped dose-response relationship with a maximal efficacy at 10 nM nefiracetam (Fig. 2B, E).

Although the mechanism that underlies the lack of NMDA response of bipolar neurons to the potentiating action of 10 nM nefiracetam remains to be seen, there is a possibility that bipolar neurons have a much lower sensitivity to nefiracetam than multipolar neurons. However, this possibility was ruled out by the experiments using bipolar neurons that showed no potentiation by nefiracetam even at higher concentrations up to 1000 nM (Fig. 2B, F).

Nefiracetam Potentiation Is Independent of NMDA Concentration.

At least two possible mechanisms of nefiracetam potentiation of NMDA currents are conceivable. One is an increase in the affinity of the receptor for NMDA causing a shift of NMDA dose-response curve in the direction of lower concentrations of NMDA. The other possibility to potentiate NMDA currents is an increase in current amplitude even at high concentrations of NMDA that produce a saturating response, without causing a shift of NMDA dose-response curve. The dose-response relationships for NMDA-induced currents before and during bath perfusion of 10 nM nefiracetam are illustrated in Fig. 3A. The peak current amplitudes normalized to the maximum control current induced by 1000 µM NMDA were fitted by a sigmoid curve with an EC50 of 36.9 ± 0.3 µM and a Hill coefficient of 0.58 ± 0.07 (Fig. 3A, ○, n = 4). After a 10-min bath perfusion of 10 nM nefiracetam, the maximal current amplitude induced by 1000 µM NMDA was 148.2 ± 7.5% of the control maximum, an EC50 was 28.2 ± 0.2 µM, and a Hill coefficient was 0.66 ± 0.08 (Fig. 3A, ●, n = 4). Thus, nefiracetam caused a slight shift of NMDA dose-response curve in the direction of lower concentrations, and a large increase in maximal current amplitude resulting in an increase in the saturating response evoked by 1000 µM NMDA. Therefore, the latter mechanism is mainly responsible for nefiracetam potentiation of NMDA currents.

The current amplitudes relative to the control during perfusion with nefiracetam are plotted as a function of NMDA concentrations in Fig. 3B. Nefiracetam at 10 nM significantly potentiated currents at all NMDA concentrations in a manner independent of NMDA concentrations.

Nefiracetam-Glycine Interactions at the NMDA Receptor.

One of the possible sites of action of nefiracetam in the NMDA receptor is the glycine binding site. To examine this possibility, interactions of glycine and nefiracetam at the NMDA receptor were studied in the absence of Mg2+ ions in

Fig. 1. Nefiracetam potentiates NMDA-induced currents in multipolar-type neurons (30–60 µm in diameter) but not in bipolar-type neurons (15–30 µm in diameter). Currents were evoked at a holding potential of -70 mV by 250-ms applications of 30 µM NMDA via a U-tube system at an interval of 1 min. A, currents recorded from a multipolar neuron before (a), during (b), and after (c) bath application of 10 nM nefiracetam. B, time course of changes (mean ± S.D., n = 5) in peak current amplitude before, during, and after bath application of 10 nM nefiracetam in multipolar neurons. C, currents recorded from a bipolar neuron before (a), during (b), and after (c) bath application of 10 nM nefiracetam. Nefiracetam had no effect on the current. D, time course of changes (mean ± S.D., n = 5) in peak current amplitude before, during, and after bath application of 10 nM nefiracetam in bipolar neurons. Nefiracetam had no effect on the current.
the external solution. The NMDA dose-response relationship in the presence of 3 μM glycine in the external solution is shown in Fig. 3A (▲). The current was greatly potentiated by glycine, and the data were fitted by a sigmoid curve with the maximal current of 159.8 ± 11.7% of the control maximum, an EC_{50} of 26.7 ± 0.3 μM, and a Hill coefficient of 0.75 ± 0.05 (Fig. 3A, ▲, n = 4). Addition of 10 nM nefiracetam to the solution containing 3 μM glycine resulted in a decrease in current amplitude from the level of 3 μM glycine alone. The current amplitudes were fitted by a sigmoid curve with the maximal current of 196.5 ± 9.2% of the control maximum, an EC_{50} of 30.0 ± 0.2 μM, and a Hill coefficient of 0.70 ± 0.07 (Fig. 3A, △, n = 4). Therefore, the ability of 3 μM glycine in potentiating NMDA currents was attenuated by 10 nM nefiracetam.

The interactions between glycine and nefiracetam at the NMDA receptor are more clearly shown in the experiments in which nefiracetam was tested at various concentrations (Fig. 4). After confirming the potentiating action of 3 μM glycine on NMDA currents, nefiracetam was applied to the bath at various concentrations ranging from 1 to 10,000 nM. The glycine-induced potentiation was reduced with increasing concentration of nefiracetam, almost disappeared at 100 nM nefiracetam, and was reversed at 1,000 and 10,000 nM nefiracetam showing slight suppression beyond the level of control. These effects were reversible after washing with a solution free of glycine and nefiracetam. These results lead to the suggestion that nefiracetam binds to the glycine binding site of the NMDA receptor.

The interactions between glycine and nefiracetam were further studied using various concentrations of glycine. A series of experiments illustrated in Fig. 5A indicated that although glycine, when applied via the U-tube, potentiated NMDA currents in a concentration-dependent manner until the current amplitude reached a maximum at 3000 nM glycine. The same protocol, but in the presence of 10 nM nefiracetam in the bath, caused attenuation of current amplitude over and above that at 100 nM glycine. Thus, nefiracetam at 10 nM abolished potentiation caused by high concentrations of glycine at 1000 and 3000 nM. These results suggest that nefiracetam binds to the glycine site with higher affinity but with lesser efficacy than glycine.

The data shown in Fig. 5A appeared to be inconsistent with the partial agonist hypothesis, because glycine at high concentrations did not overcome the effect of nefiracetam. However, in this experiment, glycine was coapplied via the U-tube with glutamate. There might not be sufficient time for glycine to compete with bath-applied nefiracetam for the glycine site, which is consistent with our previous conclusion that nefiracetam is a more potent but less efficacious agonist than glycine.

To further pursue this problem, the experiment shown in new Fig. 5B was carried out under an equilibrium condition where glycine was applied both through the bath and via the U-tube. Thus, glycine would have ample time to compete with nefiracetam for the glycine site. Glycine at high concentrations was indeed able to compete with nefiracetam, and the maximum effect of glycine was not reduced by the presence of nefiracetam.

7-Chlorokynurenic Acid Abolishes Nefiracetam Potentiation of NMDA Currents. The aforementioned results suggest that nefiracetam acts on the glycine binding site of the NMDA receptor thereby potentiating NMDA-induced currents. To prove the validity of this hypothesis, 7-chlorokynurenic acid (7-ClKCN), which is known to be a glycine binding site antagonist was used. 7-ClKCN applied to the bath at a concentration of 300 nM did not change the amplitude of current evoked by 30 μM NMDA (Fig. 6A, a and b). However, 7-ClKCN at 1 μM greatly suppressed the NMDA current (Fig. 6A, c). In the presence of 1 μM 7-ClKCN in the bath, nefiracetam at 10 nM no longer caused potentiation of NMDA current (Fig. 6A, d). Washing with drug-free solution restored the current (Fig. 6A, e), and bath perfusion of 10 nM nefiracetam again potentiated the current (Fig. 6A, f) reversibly (Fig. 6A, g). Thus, it was concluded that nefiracetam binds to the glycine site of the NMDA receptor thereby causing a potentiation of NMDA-induced currents.

To examine the interaction between nefiracetam and 7-ClKCN in a more detailed manner, the dose-response relationship for 7-ClKCN to inhibit NMDA currents was deter-
mined before and during application of 10 nM nefiracetam (Fig. 7). The IC_{50} values estimated from the dose-response relationships were 295.6 ± 31.1 and 467.6 ± 24.9 nM in the absence and presence of nefiracetam, respectively. Thus, the nefiracetam-induced shift of the dose-response curve in the direction of higher concentrations is consistent with the competitive interaction of nefiracetam and 7-ClKN at the glycine site.

Effects of Nefiracetam on AMPA and Kainate Currents. Besides the NMDA current, rat cortical neurons in primary culture generated currents in response to U-tube application of AMPA or kainate. AMPA- and kainate-induced currents were not significantly affected by nefiracetam. Figure 8, A and B, shows that 10 nM nefiracetam slightly potentiated currents induced by 30 μM AMPA, but the effect was not significant. Figure 8, C and D, shows that currents induced by 30 μM kainate were not affected at all by 10 nM nefiracetam. It was concluded that among the three subtypes of glutamate receptors, the AMPA and kainate receptors are not responsive to any modulating action of nefiracetam.

Discussion

We have previously reported that nefiracetam augments the α4β2-type nACh receptor currents in a highly potent (≈1 nM) and efficacious (to ∼200% of control) manner (Zhao et al., 2001). The present study demonstrates that the NMDA receptor is another target highly sensitive to the potentiating action of nefiracetam. NMDA-induced currents in rat cortical neurons in primary culture were potentiated by nefiracetam following a bell-shaped dose-response curve with the maximal potentiation occurring at 10 nM. Evidence was obtained to indicate that nefiracetam acted on the glycine binding site of the NMDA receptor. Nefiracetam had little or no effect on AMPA- or kainate-induced currents.

It is well known that in the brain of Alzheimer’s patients the NMDA receptor as well as the nACh receptor is down-regulated. The role of nACh receptors in Alzheimer’s patients received much attention (Giacobini, 2000), and four anticholinesterases have been developed into clinical use to improve cognition/learning/memory of the patients through stimulation of the cholinergic system. However, these anticholinesterases cause side effects characteristic of cholinesterase inhibition and the therapeutic effects are not long-lasting. Thus, other ways of improving the patient’s conditions need to be sought. Direct stimulation of nACh receptors without inhibiting cholinesterase seems to be a logical approach, and nefiracetam exerts exactly that action. It also seems helpful if a drug stimulates both nACh and NMDA receptors. We have now found that nefiracetam potentiates NMDA and ACh currents. Although overstimulation of NMDA receptors would cause cell death via excess influx of Ca^{2+} ions, down-
regulation of NMDA receptors would have deteriorative effects especially on learning and memory (Brown et al., 1997). Thus, potentiation of NMDA receptor activity, which is suppressed in the brain of Alzheimer’s patients, is deemed therapeutic up to the normal level.

Nefiracetam was found to interact with the glycine binding site of the NMDA receptor. As expected from its higher affinity but lesser efficacy than glycine, nefiracetam potentiates NMDA currents in the nominal absence of glycine, or in the presence of low concentrations of glycine, it suppresses the NMDA currents augmented by higher concentrations of glycine. This will prevent the NMDA receptor from being overactivated. In fact, d-cycloserine, a partial glycine site agonist, is known to improve implicit memory performance of words in Alzheimer’s patients (Schwartz et al., 1996) and reversal learning of rat in the water maze (Rickkinen et al., 1998). Thus, nefiracetam and d-cycloserine, as the glycine site partial agonists, share the common mechanism of action with respect to stimulation of NMDA receptors.

The mechanism underlying a bell-shaped dose-response curve for nefiracetam potentiation of NMDA currents is unknown. However, a similar bell-shaped dose-response curve was found in nefiracetam potentiation of ACh currents (Zhao et al., 2001), in nefiracetam potentiation of high voltage-gated calcium channel currents (Yoshii and Watabe, 1994), in GABA release (Watabe et al., 1993), and even in improve-
ment of learning and memory in rat behavioral experiments (Sakurai et al., 1989). The mechanism of bell-shaped dose-response curves awaits further experimentation.

Morphological features of the cells dissociated from 15-day gestational age rat fetal cortex after 4 to 6 weeks of culture were found similar to those of cortical neurons in situ; neurons were characterized by their cell forms such as pyramidal, multipolar, fusiform, etc. (Kriegstein and Dichter, 1983). Responses to nefiracetam as revealed in the present study were different depending on the cell type. NMDA currents recorded from pyramidal and multipolar neurons were potentiated by nefiracetam, whereas the currents from bipolar neurons were not affected by nefiracetam. One possible reason for this differential action is the differences in NMDA receptor subtypes. The NMDA receptor comprises one or more of the four NR2 subunits, NR2A, NR2B, NR2C, and NR2D, together with at least one NR1 subunit (Sucher et al., 1996). The NR1 and NR2B subunits display high levels of expression during the 1st week of development. In contrast, the expression of the NR2A subunit increases gradually reaching the matured levels by 21 days in culture (Li et al., 1998). Thus, the subunit composition of the NMDA receptor including the NR2A and NR2B subunits may be different between pyramidal/multipolar-type and bipolar-type neurons exhibiting differential sensitivity to nefiracetam.

Another possibility is that the second messenger system, specifically that related to phosphorylation and dephosphorylation, might be different among these neurons. The NMDA receptor is known to be modulated by phosphorylation. Further experiments are needed to explore this possibility.

Fig. 7. Nefiracetam 10 nM shifts the dose-response relationship for 7-chlorokynurenic acid inhibition of currents induced by 30 μM NMDA in the direction of higher concentration. Multipolar neurons IC₅₀ values: 295.6 ± 31.1 nM in control (n = 4); 467.6 ± 24.9 nM in nefiracetam (n = 4). 7-CIKN was applied both to the bath and via U-tube, and nefiracetam was applied to the bath. Glycine was added to the external solution at a concentration of 10 nM.

Fig. 8. Effects of bath perfusion of 10 nM nefiracetam on currents evoked by 30 μM AMPA (A and B, n = 4) and 30 μM kainate (C and D, n = 4), in multipolar neurons. Holding potential ~70 mV. Neither AMPA-induced nor kainate-induced currents were significantly affected by 10 nM nefiracetam.
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References


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