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Nootropic Drug Modulation of Neuronal Nicotinic Acetylcholine Receptors in Rat Cortical Neurons

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ABSTRACT

Nefiracetam (DM-9384) is a new pyrrolidone nootropic drug being developed for the treatment of Alzheimer's type and poststroke vascular-type dementia. Because the cholinergic system plays an important role in cognitive functions and Alzheimer's disease dementia, the present study was conducted to elucidate the mechanism of action of nefiracetam and aniracetam on neuronal nicotinic acetylcholine receptors (nnAChRs). Currents were recorded from rat cortical neurons in long-term primary culture using the whole-cell, patch-clamp technique. Two types of currents were evoked by acetylcholine (ACh): α-bungarotoxin-sensitive, α7-type currents and α-bungarotoxin-insensitive, $\alpha4\beta2$-type currents. Although nefiracetam and aniracetam inhibited α7-type currents only weakly, these nootropic agents potentiated $\alpha4\beta2$-type currents in a very potent and efficacious manner. Nefiracetam at 1 nM and aniracetam at 0.1 nM reversibly potentiated $\alpha4\beta2$-type currents to 200 to 300% of control. Nefiracetam at very high concentrations (~10 μM) also potentiated $\alpha4\beta2$-type currents but to a lesser extent, indicative of a bell-shaped dose-response relationship. Nefiracetam markedly increased the saturating responses induced by high concentrations of ACh. However, human $\alpha4\beta2$ subunits expressed in human embryonic kidney cells were inhibited rather than potentiated by nefiracetam. The specific protein kinase A inhibitors (H-89, KT5720, and peptide 5-24) and protein kinase C inhibitors (chelerythrine, calphostin C, and peptide 19–63) did not prevent nefiracetam from potentiating $\alpha4\beta2$-type currents, indicating that these protein kinases are not involved in nefiracetam action. The nefiracetam potentiating action was not affected by 24-h pretreatment of neurons with pertussis toxin, but was abolished by cholera toxin. Therefore, G_s proteins, but not G_i/G_o proteins, are involved in nefiracetam potentiation. These results indicate that nnAChRs are an important site of action of nefiracetam and G_s proteins may be its crucial target.

Nootropic drugs may be classified into two groups, pyrrolidone derivatives and cholinergic agonists/anticholinesterases. A limited number of oxopyrrolidine acetic acid (racetam) derivatives have been developed into clinical use as cognitive enhancers (Gouliaev and Senning, 1994). Aniracetam has been used as a cognitive enhancer. Nefiracetam (DM-9394) is a new pyrrolidine nootropic drug and, after extensive animal behavioral experiments (Nabeshima, 1994; Nabeshima et al., 1994), it is undergoing preclinical and clinical tests as a cognition-enhancing drug in Alzheimer's disease and poststroke dementia.

The mechanisms of action of aniracetam and nefiracetam remain largely to be seen. Aniracetam is known to modulate the activity of glutamatergic system. Glutamate-evoked responses were augmented, fast excitatory postsynaptic potential and excitatory postsynaptic current were potentiated, and their desensitization was slowed, and the open time of single channels was lengthened (Vyklicky et al., 1991; Barbour et al., 1994; Partin et al., 1996). These effects seemed to be exerted via AMPA receptors but only at high concentrations (1–5 mM) (Ito et al., 1990; Isaacson and Nicoll, 1991; Tang et al., 1991). However, no effect was observed on *N*-methyl-D-aspartate receptors (Ito et al., 1990).

Aniracetam was effective in increasing high voltage-gated calcium channel currents (Yoshii and Watabe, 1994). Nefiracetam also augmented high voltage-gated N/L-type calcium channel currents at micromolar concentrations (~1 μM) via interactions with G proteins (Yoshii and Watabe, 1994; Yoshii et al., 1997). The GABAergic system is also modulated by nefiracetam (Huang et al., 1996). Depending on GABA concentration, GABA-induced currents were either potentiated or inhibited by 3 to 1000 μM nefiracetam, and protein kinase A (PKA) and G proteins, but not protein kinase C (PKC), were deemed involved in nefiracetam modulation of the $GABA_A$ system. The effects of nefiracetam on neuronal nicotinic acetylcholine receptors (nnAChRs) in

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ABBREVIATIONS: GABA, γ-aminobutyric acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; PKA, protein kinase A; PKC, protein kinase C; nnAChR, neuronal nicotinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; AChR, acetylcholine receptor; ACh, acetylcholine; α-BuTX, α-bungarotoxin; HEK, human embryonic kidney; DHβE, dihydro-β-erythroidine.

PC12 cells were similar to those on the $GABA_A$ receptor (Oyaizu and Narahashi, 1999).

The cholinergic system seems to be an important target site of nefiracetam. A recent study by Nishizaki et al. (1998) has indeed demonstrated an important feature of nefiracetam-acetylcholine receptor interactions. Using *Torpedo californica* nicotinic AChRs expressed in *Xenopus laevis* oocytes, nefiracetam at 0.01 to 0.1 μM caused a short-term depression of ACh-induced currents and a long-term potentiation at higher concentrations (1 to 10 μM). Nefiracetam depression was caused by the activation of pertussis toxin-sensitive, G protein-regulated PKA activity. On the contrary, nefiracetam potentiation was caused by the activation of Ca^{2+}-dependent PKC. Nefiracetam also induced long-term potentiation-like facilitation of hippocampal synaptic transmission (Nishizaki et al., 1999). Human $\alpha4\beta2$ and $\alpha7$ AChRs expressed in *X. laevis* oocytes were potentiated by 10 nM nefiracetam, but not by aniracetam (Nishizaki et al., 2000).

The nicotinic acetylcholine receptor (nAChR) gene family may be classified into three groups: 1) nAChRs of skeletal muscles and fish electric organs; 2) α-bungarotoxin (α-BuTX)-sensitive nnAChRs; and 3) α-BuTX-insensitive nnAChRs (Lindstrom, 1996). nAChRs of fetal muscle comprise five subunits $(\alpha1)_2\beta1\gamma\delta$, and those of adult muscle have five subunits with somewhat different combinations of $(\alpha1)_2\beta1\epsilon\delta$ (Changeux, 1990).

α-BuTX-sensitive nnAChRs are pentameric homomers and are composed of $\alpha7$, $\alpha8$, or $\alpha9$ subunits (Couturier et al., 1990; Seguela et al., 1993; Elgoyhen et al., 1994). The $\alpha7$ subunit is a predominant α-BuTX-sensitive nnAChR in the mammalian brain. α-BuTX-insensitive nnAChRs have a pentameric structure consisting of a combination of $\alpha2$, $\alpha3$ or $\alpha4$ subunits with $\beta2$, $\beta4$ and/or $\alpha5$ subunits (Sargent, 1993). There is general agreement that the nnAChRs of mammalian brain have predominantly $\alpha4$ and $\beta2$ subunits. The $\alpha3$ subunit, especially in the form of $\alpha3\beta4$, seems to be located in ganglia (Whiting and Lindstrom, 1988; Flores et al., 1996). The $\alpha4\beta2$ and $\alpha7$ nnAChRs in the brain are deemed to play an important role in cognitive function (Alkondon et al., 2000).

Because nicotinic AChRs seem to play an important role in aniracetam and nefiracetam action, and also because most data are obtained in receptors expressed in host cells, it is critically important to analyze the effects of these drugs on the nnAChRs of native brain neurons, $\alpha4\beta2$-type and $\alpha7$-type receptors in particular. It should be emphasized that the receptors recombinantly expressed in various host cells such as *X. laevis* oocytes and human embryonic kidney (HEK) cells do not necessarily behave physiologically and pharmacologically in the same manner as native neurons with the same receptors (Cooper and Millar, 1997; Lewis et al., 1997; Sivilotti et al., 1997; Sweileh et al., 2000).

We found in the present study that both nefiracetam and aniracetam potently augmented the $\alpha4\beta2$-type currents and weakly inhibited the $\alpha7$-type currents in rat cortical neurons. The $\alpha4\beta2$ receptors expressed in HEK cells were inhibited rather than potentiated by nefiracetam. Nefiracetam action seems to be exerted via G_s proteins.

Materials and Methods

Cerebral Cortical Neuron Preparations. Rat cortical 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 a 17-day pregnant Sprague-Dawley rat under methoxyflurane anesthesia. Small wedges of frontal cortex were excised and subsequently incubated in phosphate buffer solution containing 0.25% (w/v) trypsin (Type XI; Sigma, St. Louis, MO) for 20 min at 37°C. 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 (Life Technologies, Gaithersburg, MD) 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 cocultures were maintained in a humidified atmosphere of 90% air and 10% CO_2 at 34°C. Cells cultured for 4 to 7 weeks were used for nnAChR experiments.

HEK tsA201 Cell Culture. The HEK tsA201 cell line stably expressing the human nnAChR $\alpha4\beta2$ subunit combination was prepared at the University of Pennsylvania. HEK cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), 6% iron-supplemented calf serum (Sigma), and 100 μg/ml G418 (Mediatech, Herndon, VA). For patch-clamp experiments, HEK cells were plated on glass coverslips coated with poly-L-lysine and cultured at 35°C with 7% CO_2 and 93% air in an incubator for 3 to 5 days before an experiment.

Solutions for Current Recording. The external solution for whole-cell recordings of ACh-induced currents contained 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM HEPES, 10 mM HEPES sodium, and 10 mM D-glucose. Tetrodotoxin (100 nM) was added to eliminate the voltage-gated sodium channel currents. Atropine sulfate (20 nM) was added to block the muscarinic AChR currents. The pH was 7.3 and the osmolarity was adjusted to 300 mOsM with D-glucose. The internal pipette solution contained 140 mM Cs gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, 2 mM ATP-Mg^{2+}, and 0.2 mM GTP-Na^+. The pH was adjusted to 7.3 with CsOH, and the osmolarity was adjusted to 290 to 300 mOsM with D-glucose.

Whole-Cell Current Recordings. Ionic currents were recorded using the whole-cell, patch-clamp technique (Hamill et al., 1981) at room temperature (21–22°C). Pipette electrodes were made from 1.5-mm (outer diameter) borosillicate glass capillary tubes with a resistance of 2 to 3 MΩ when filled with the standard internal solution. The membrane potential was clamped at −70 mV. 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 (Axon Instruments, Foster City, 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 means ± S.D., and n represents the number of the cells examined.

Chemicals. ACh (Sigma) was first dissolved in distilled water to make stock solutions. The G_i/G_o protein inhibitor pertussis toxin, the G_s protein stimulator cholera toxin, the voltage-gate sodium channel blocker tetrodotoxin, the $\alpha7$-type nnAChR blocker α-BuTX, the $\alpha4\beta2$-type nnAChR blocker dihydro-β-erythroidine (DHβE), and the muscarinic AChR blocker atropine sulfate were purchased from Sigma. PKA inhibitors including peptide 5-24, H89, KT5720, and PKC inhibitors including peptide 19-36, chelerythrine chloride and calphostin C were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Nefiracetam [DM-9384; *N*-(2,6-dimethylphenyl)-2-(2-oxo-*l*-pyrrolidinyl) acetamide] (Fig. 1) was provided by Daiichi Pharmaceutical Company (Tokyo, Japan) and first dissolved in distilled water as stock solutions. Aniracetam [1-(4-methoxybenzoyl)-2-pyrrolidinone (Fig. 1)] was purchased from Sigma and was dissolved in dimethyl sulfoxide (Sigma) as stock solutions. These stock solutions of nootropic drugs were stored at 4°C and diluted to prepare test solutions with the standard external solution shortly before the

experiments. The final concentrations of dimethyl sulfoxide in test solutions were 0.1% (v/v) or less, which had no effect on the ACh-activated currents.

Drug Application. Two methods for drug application were used: one was application via a U-tube and the other was perfusion through the bath. The ACh solution was applied through a fast U-tube application system (Fenwick et al., 1982) controlled by computer-operated magnetic valves. When one of the valves was open, the ACh solution was allowed to bypass the chamber. When it was closed, the ACh solution was ejected through the hole of the U-tube, which was located close to the cell. At the same time, another valve controlling the suction tube was opened, allowing the test solution to be sucked away quickly. The external solution surrounding the cell could be completely changed with the ACh solution within 30 to 40 ms. Test drugs were added to the external solution and continuously perfused to the recording chamber via a glass syringe and Teflon-tube.

Results

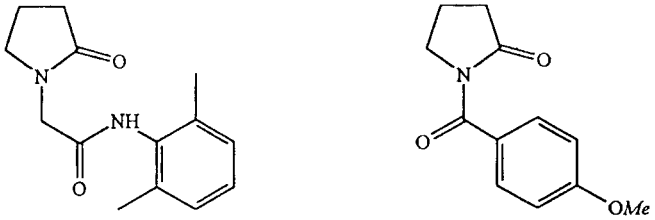
Two Types of Currents Evoked by ACh in Cortical Neurons. Rat cortical neurons in long-term primary culture expressed nnAChRs. To record the currents mediated by nnAChRs, 100 nM tetrodotoxin was added to external solutions to inhibit the voltage-gated Na^+ channel, 1 mM Mg^{2+} to block the *N*-methyl-D-aspartate receptors, and 20 nM atropine to suppress the neuronal muscarinic AChRs. Two types of currents were induced by ACh: rapidly desensitizing, α7-type currents, which were irreversibly blocked by α-BuTX (Fig. 2A), and slowly desensitizing, $\alpha4\beta2$-type currents, which were insensitive to the blocking action of α-BuTX but were reversibly blocked by DHβE (Fig. 2B), as described by Aistrup et al. (1999). Most neurons had both α7-type and $\alpha4\beta2$-type nnAChRs exhibiting a rapidly decaying current followed by a slowly decaying component. Therefore, to separately record the two types of nnAChR currents, 70 nM DHβE or 100 nM α-BuTX was added to the external solution to suppress $\alpha4\beta2$-type or α7-type currents, respectively.

Irreversible Inhibition of α7-type Currents by Aniracetam and Nefiracetam. Aniracetam and nefiracetam inhibited α7-type currents weakly and irreversibly. α7-Type currents were induced by 0.5-s, 300 μM ACh pulses applied through the U-tube device at 1-min intervals while the membrane was held at -70 mV. The EC_{50} value for ACh activation was 344 $\pm$ 30 μM (n = 7). The peak amplitude of currents evoked by ACh ran down slowly at a rate of 14.7 $\pm$ 2.3% over a period of 50 min from the beginning of recording (n = 4) (Fig. 3B, control).

When 10 μM aniracetam was perfused after several stable control recordings were obtained, the peak amplitude of α7-type current was gradually decreased by 20.8 $\pm$ 6.7% over a period of 20 min (n = 4), whereas the run-down of control current in 20 min was 3.8 $\pm$ 3.1% (n = 4) (Fig. 3A). This inhibitory effect of aniracetam on α7-type currents was irreversible after washout with drug-free external solution up to 30 min (Fig. 3A).

Nefiracetam at 1, 10, and 100 μM also inhibited α7-type currents weakly (Fig. 3B). After perfusion of 1, 10, and 100 μM nefiracetam for 10 min each, and after corrections for the run-down, the currents were decreased by 2.8, 8.7, and 20.1%, respectively (n = 4). Thus, nefiracetam exerts a weak inhibitory action on the α7-type current. The ACh dose-response curve in the presence of 10 μM nefiracetam was slightly shifted from an EC_{50} value of 344 $\pm$ 30 μM to 445 $\pm$ 41 μM with no changes in the maximum response and Hill coefficients (n = 7) (data not shown).

Reversible Potentiation of $\alpha4\beta2$-type Currents by Nefiracetam. In contrast to α7-type currents, $\alpha4\beta2$-type currents were potently and reversibly augmented by nefiracetam. $\alpha4\beta2$-Type currents were induced by 10 μM ACh at a membrane potential of -70 mV. The peak amplitude of $\alpha4\beta2$-type currents was gradually enhanced during the first 4 to 6



Nefiracetam (DM-9384)
$C_{14}H_{18}N_2O_2$

Aniracetam
$C_{12}H_{13}NO_3$

Fig. 1. The chemical structures of the nootropic drug, nefiracetam (DM-9384), and aniracetam.

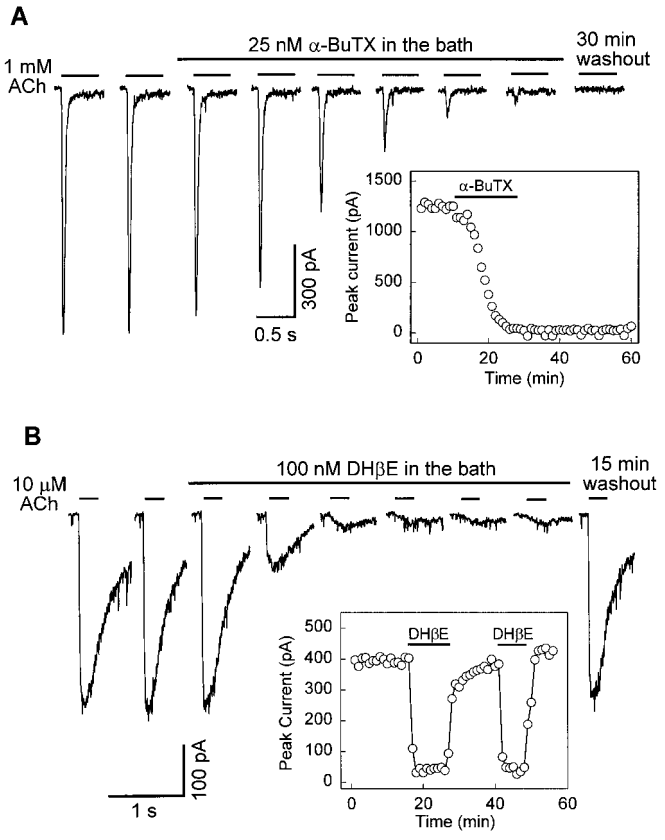


Fig. 2. α7-type and $\alpha4\beta2$-type currents in cortical neurons. A, in the presence of 70 nM DHβE in the external solution to block $\alpha4\beta2$-type nnAChRs, ACh at 1 mM evoked fast-desensitizing currents that were irreversibly suppressed by α-BuTX at a holding potential of -70 mV. B, in the presence of 100 nM α-BuTX in the external solution to block α7-type nnAChRs, ACh at 10 μM evoked slow-desensitizing currents which were reversibly suppressed by DHβE at a holding potential of -70 mV.

min of bath perfusion of 1 nM nefiracetam. The potentiation remained stable at about 250% of the control during the next 40-min perfusion of nefiracetam and was reversible after washout with drug-free external solution (Fig. 4A and B). The minimum effective concentration of nefiracetam was 0.1 nM, at which only a small potentiation was observed (Fig. 4C). Nefiracetam at higher concentrations (10 nM, 1 μM, and 10 μM) also potentiated the $\alpha4\beta2$-type currents, but there was a tendency for the potentiation to lessen (Fig. 4C). In some neurons, nefiracetam at 1 μM potentiated the current with a fast transient increase followed by a decrease to a steady level (Fig. 4D). Thus, a bell-shaped dose-response relationship is indicated.

To investigate whether the nefiracetam-induced potentiation of $\alpha4\beta2$-type currents depended on repetitive ACh activation of nnAChRs, ACh pulses were applied to the neuron at 10-min intervals. During a 20-min bath application of nefiracetam (Fig. 5), the degrees of current potentiation were similar to those produced by consecutive ACh stimulation applied at 1-min intervals, indicating that the nefiracetam-induced potentiation of $\alpha4\beta2$-type currents does not depend on the opening of the nnAChR channels.

Nefiracetam Potentiation as a Function of ACh Concentration in $\alpha4\beta2$-Type Receptors. Ligand-activated currents may be potentiated by a drug because of a shift of the dose-response curve in the direction of lower concentrations of ligand or because of an increase in current responses irrespective of ligand concentrations. To distinguish these possibilities, nefiracetam potentiation of $\alpha4\beta2$-type currents was examined as a function of ACh concentration. ACh dose-response relationships were obtained by plotting currents

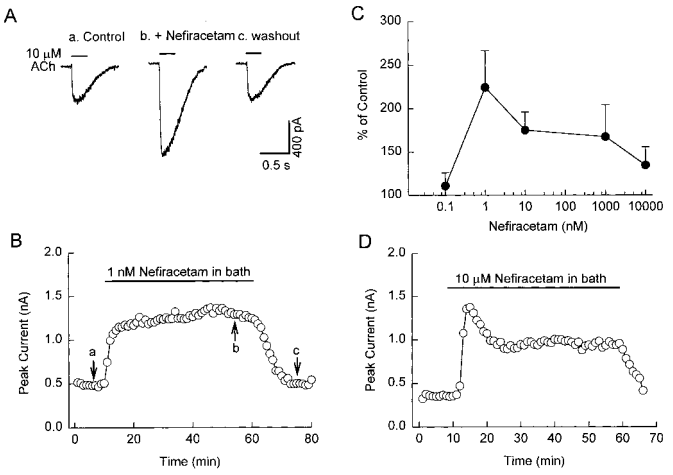


Fig. 4. Nefiracetam reversibly potentiates the $\alpha4\beta2$-type currents in cortical neurons. A, sample traces, and B, time course of currents induced by 10 μM ACh at a holding potential of -70 mV before, during and after bath application of 1 nM nefiracetam. The peak current amplitude was gradually increased in 10 min following nefiracetam bath application and then was kept at a relatively stable level during a 45-min nefiracetam treatment. C, the bell-shaped dose-response curve for nefiracetam potentiation of currents evoked by 10 μM ACh measured after 15 min of treatment with nefiracetam in the bath. The percentages of current increase induced by nefiracetam at 0.1, 1, 10, 1,000, and 10,000 nM were 10.7 $\pm$ 15.3%, 124.6 $\pm$ 41.9%, 75.2 $\pm$ 21.0%, 67.7 $\pm$ 36.9%, and 34.7 $\pm$ 21.2%, respectively (mean $\pm$ S.D., n = 5–9). D, the same as B but with 10 μM nefiracetam.

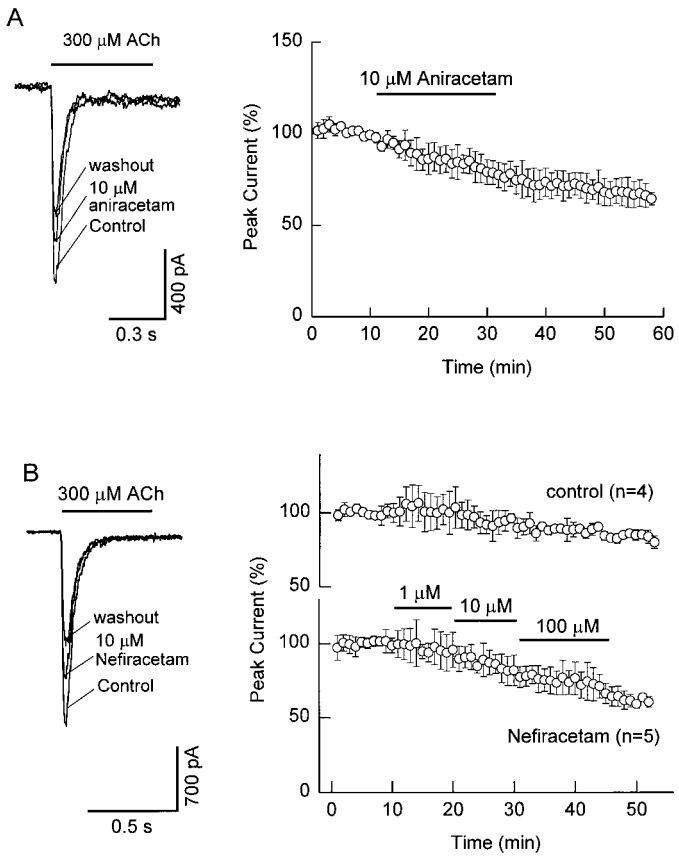


Fig. 3. $\alpha7$-Type currents are weakly suppressed by aniracetam and nefiracetam in cortical neurons. $\alpha7$-Type currents were induced by 0.5 s 300 μM ACh pulses at 1-min intervals at a holding potential of -70 mV. A, sample records of currents before, during and after treatment with 10 μM aniracetam in the bath, and the time course of the inhibitory effect (n = 4). This inhibitory effect was irreversible after washout with aniracetam-free external solution for 30 min. B, in the presence of nefiracetam at 1 μM, 10 μM, and 100 μM in the bath, the peak amplitude of currents were slightly inhibited (n = 5). Data represent the mean $\pm$ S.D.

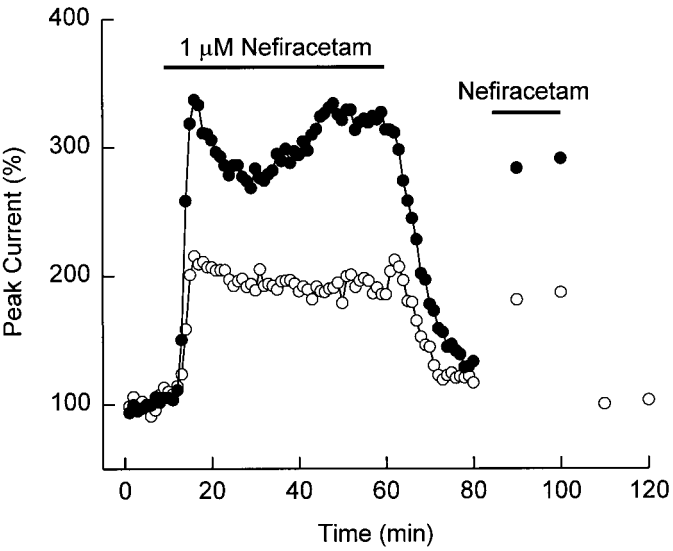


Fig. 5. Use-independent nefiracetam potentiation of $\alpha4\beta2$-type nnAChRs in two cortical neurons. To investigate the possibility of the use dependence of the potentiating effect of nefiracetam, two ACh pulses were given at a 10-min interval during 20-min reapplication of nefiracetam after washout of the potentiation response evoked by 10 μM ACh. The potentiating responses were similar to those produced by consecutive ACh stimulation at 1-min intervals, indicating that the potentiation does not depend on the activation of the nnAChR. The closed and open circles represent two separate experiments.

induced by ACh concentrations ranging from 0.1 to 1000 μM. The peak current amplitude normalized to the maximum current (I_{max}) induced by 1000 μM ACh was fitted by a sigmoid curve with I_{max} = 100.0 ± 2.0%, EC_{50} = 2.0 ± 0.2 μM, and Hill coefficient (n_H) = 0.83 ± 0.07 (Fig. 6A, ●). After a 10-min bath application of 10 nM nefiracetam, the ACh-induced currents were recorded from the same neurons using the identical protocol. The peak current amplitudes normalized to the control maximum current were fitted by a curve with I_{max} = 183.6 ± 6.7%, EC_{50} = 1.2 ± 0.3 μM, and n_H = 0.60 ± 0.07 (Fig. 6A, ○). Nefiracetam potentiation occurred even at the highest ACh concentration that generated a saturated response. The potentiation could be largely accounted for by an increase in the maximal response, as indicated by the dotted line obtained by normalizing the control maximum response to the nefiracetam maximum response (Fig. 6A). Except at 0.1 μM ACh, the degree of nefiracetam potentiation was almost independent of ACh concentration (Fig. 6B).

PKA and PKC Inhibitors Do Not Block the Nefiracetam Potentiation of $\alpha4\beta2$-Type Currents. Nefiracetam has been reported to modulate the activity of high voltage-gated calcium channels, $GABA_A$ receptors, *T. californica* ACh receptors, and PC12 ($\alpha3$-type) ACh receptors via G proteins, PKA, or PKC (Yoshii and Watabe, 1994; Huang et al., 1996; Nishizaki et al., 1998, 2000; Oyaizu and Narahashi, 1999). Thus, we first examined the role of PKA and PKC in nefiracetam potentiation of $\alpha4\beta2$-type currents. Because H-89 is a membrane-permeable, selective, and potent inhibitor of PKA, it was applied to bath. $\alpha4\beta2$-Type currents were slightly enhanced by 1 μM H-89 applied to the bath indicating the effectiveness of H-89. However, the presence of H-89 did not prevent the potentiation of ACh-evoked currents caused by nefiracetam (Fig. 7A). Nefiracetam potentiation of $\alpha4\beta2$-type currents was not affected by either the selective PKA inhibitor KT5720 (Fig. 7C), or the active PKA inhibitor peptide 5-24 (Fig. 7B), which binds to the catalytic subunit of PKA and displaces the regulatory subunit (Cheng et al., 1986; Knighton et al., 1991).

Calphostin C, chelerythrine, and peptide 19-36 are highly specific inhibitors of PKC. The peptide 19-36 acts as a pseudosubstrate by binding to the active site of PKC (House and Kemp, 1987). Both peptide 19-36 and calphostin C were internally applied to the neuron via the patch pipette, whereas chelerythrine was externally applied via the bath solution. Chelerythrine at 3 μM increased the ACh current to 166.2 ± 26.6% (n = 6) of the control. Because there was no true self-control for the internally applied inhibitor, the ACh current recorded during the first few minutes after breaking the seal was used as the control. Both peptide 19-36 and calphostin C showed some potentiation, on the order of 20 to 50%. The presence of either calphostin C or peptide 19-36 in the internal solution, or chelerythrine in the external solution, did not prevent the nefiracetam potentiation of currents

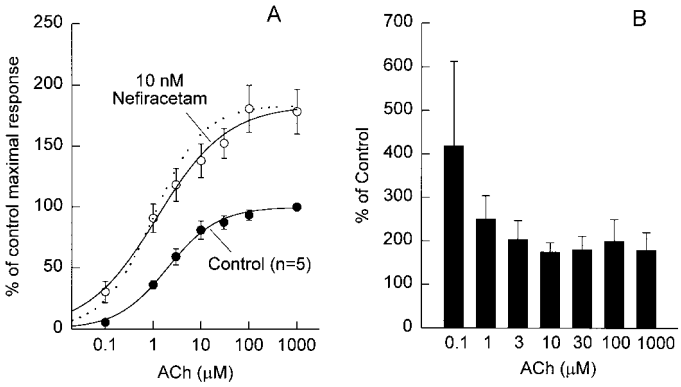


Fig. 6. Effects of nefiracetam on the ACh dose-response relationship of the $\alpha4\beta2$-type nnAChRs in cortical neurons. Currents evoked by 0.1, 1, 3, 10, 30, 100, and 1000 μM ACh in the absence and presence of 10 nM nefiracetam in the bath are plotted. The currents were first induced by 250 ms ACh pulses at 90-s intervals at a holding potential of −70 mV. Then after a 10-min bath application of nefiracetam, ACh-induced currents were recorded using the identical protocol in the same neurons. A, the current amplitude was normalized to that induced by 1000 μM ACh in the absence of nefiracetam. Control data (●) were fitted by a sigmoid curve with I_{max} = 100.0 ± 2.0%, EC_{50} = 2.0 ± 0.2 μM, and Hill coefficient = 0.83 ± 0.07. In the presence of nefiracetam, data (○) were fitted by a curve with I_{max} = 183.6 ± 6.7%, EC_{50} = 1.2 ± 0.3 μM, and Hill coefficient = 0.60 ± 0.07. The dotted line was drawn with I_{max} = 184%, EC_{50} = 2 μM, and n_H = 0.83. B, the percentage increments of the peak amplitude of the currents induced by different concentrations of ACh in the presence of 10 nM nefiracetam. Nefiracetam potentiated the currents even with the ACh concentrations that gave the saturating responses (n = 5). Data represent the mean ± S.D.

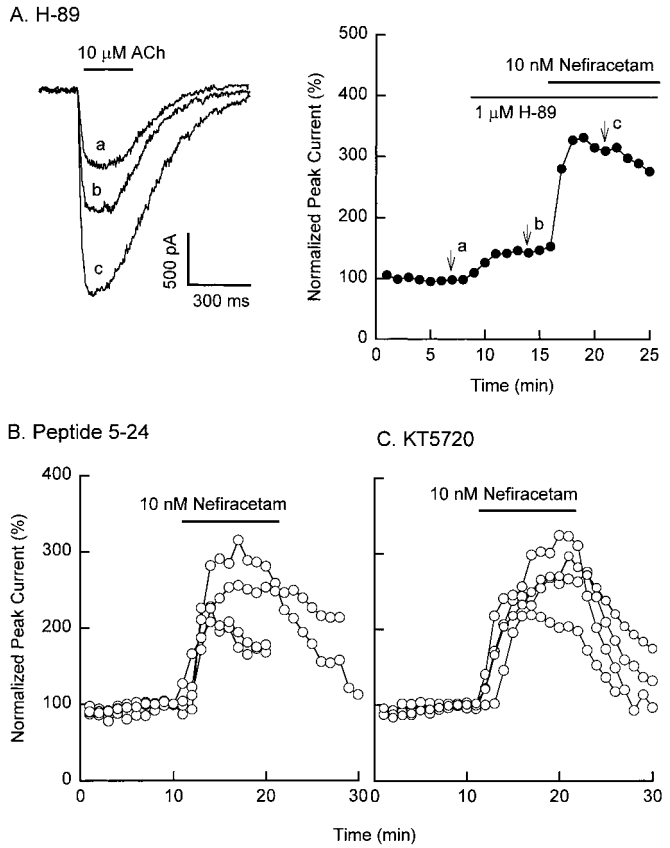


Fig. 7. PKA inhibitors do not block the nefiracetam potentiation of $\alpha4\beta2$-type currents in cortical neurons. The currents were induced by 250 ms 10 μM ACh pulses at 1-min intervals at a holding potential of −70 mV. A, sample recordings (left) and the time course (right) of changes in currents induced by ACh in the absence (a) and the presence (b) of the membrane permeable PKA inhibitor H89 (1 μM), and in the presence of H89 and 10 nM nefiracetam (c) in the external solution (n = 4). B and C, the time courses of changes in ACh-induced currents in the presence of the PKA peptide inhibitor 5-24 at 200 nM (B) and 560 nM KT5720 (C) in the internal solution. Nefiracetam at 10 nM was applied to the bath. The currents were normalized to the average peak amplitude before application of nefiracetam. Multiple open circles in B and C represent different experiments.

(Fig. 8). These data are summarized in Fig. 9, which shows treatment with PKA or PKC inhibitors do not significantly alter the nefiracetam potentiation of $\alpha4\beta2$-type currents ($P > 0.05$).

G Proteins Are Involved in the Nefiracetam Potentiation of $\alpha4\beta2$-type Currents. As described in the preceding section, G proteins were reported to play an important role in the nefiracetam modulation of various receptors and channels. However, no data are available for G protein's role in nefiracetam action on the nnAChRs of native brain neurons. Thus nefiracetam modulation was assessed in the presence of pertussis toxin or cholera toxin. Pertussis toxin and cholera toxin catalyze the ADP ribosylation of different heterotrimeric G protein catalytic G_α subunits (Gilman, 1987). Pertussis toxin catalyzes ADP ribosylation of α subunit of G_i and G_o proteins and prevents G_i heterotrimers from interacting with the receptors, blocking their coupling and activation. Because the $G_{i\alpha}$ subunits remain in the GDP-bound, inactive state, they are unable to inactivate adenylyl cyclase. In contrast, cholera toxin catalyzes ADP-ribosylation of $G_{s\alpha}$ and activates the α subunit.

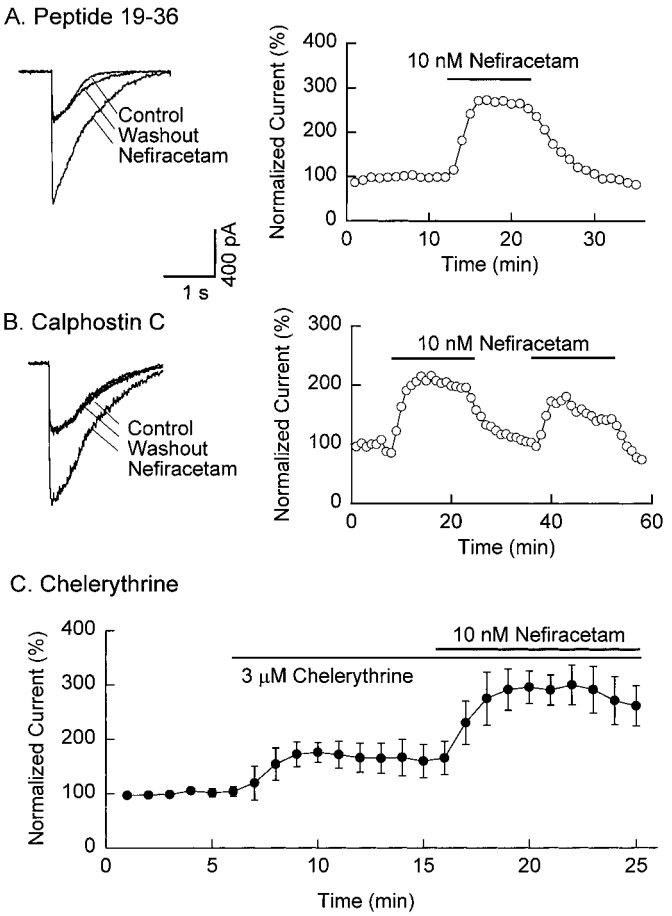


Fig. 8. PKC inhibitors do not block the nefiracetam potentiation of $\alpha4\beta2$-type currents in cortical neurons. A and B, the sample traces and changes in current amplitude induced by 10 μM ACh at a holding potential of −70 mV before, during and after bath application of 10 nM nefiracetam in the presence of the 3 μM PKC inhibitors peptide 19-36 (A) and 0.5 μM calphostin C (B) in the internal solution. C, the time course of changes in current amplitude in the absence or presence of the membrane permeable PKC inhibitor chelerythrine (3 μM) in the bath with or without 10 nM nefiracetam in the external solution ($n = 6$). Data represent the mean ± S.D.

Cortical neurons were pretreated with 200 ng/ml pertussis toxin for 24 to 26 h in culture. Nefiracetam 10 nM caused a large potentiation (250% of control) of $\alpha4\beta2$-type currents in the pertussis toxin-treated cortical neurons (Fig. 10A). Therefore, the inhibition of G_i/G_o proteins by pertussis toxin did not prevent nefiracetam potentiation of $\alpha4\beta2$-type currents, suggesting that nefiracetam does not interfere with G_i/G_o proteins.

In contrast, after pretreatment with 500 ng/ml cholera toxin for 24 to 26 h, application of 10 nM nefiracetam failed to induce any potentiation of $\alpha4\beta2$-type currents (Fig. 10B). The current amplitudes after cholera toxin treatment were comparable with those of the control and with those after pertussis toxin treatment, excluding the possibility that cholera toxin maximally potentiated the current thereby preventing the potentiating action of nefiracetam. The results suggest that G_s proteins are involved in the nefiracetam action.

Nefiracetam Inhibits Rather Than Potentiates the $\alpha4\beta2$ nAChRs Expressed in HEK Cells. To test whether nefiracetam directly acts on the receptor to potentiate ACh currents, the $\alpha4\beta2$ AChR subunits stably expressed in HEK cells were examined for their responses to nefiracetam. Slowly-desensitizing, α-BuTX-insensitive currents were induced by 10 μM ACh at a holding potential of −70 mV (Fig. 11A). In dramatic contrast to the potentiation observed in $\alpha4\beta2$-type currents recorded from native cortical neurons, a decrease in currents rather than an increase was observed by application of 10 nM nefiracetam (Fig. 11B). The effect was reversible after washing with drug-free solution. The current inhibition amounted to 26.9 ± 8.8% ($n = 10$) (Fig. 11C). This result indicates that the nefiracetam potentiation of $\alpha4\beta2$-type current in cortical neurons is via a specific intracellular messenger system that is missing in HEK cells.

Aniracetam Also Potentiates $\alpha4\beta2$-Type Currents. Aniracetam is a pyrrolidine nootropic drug and shares a similar chemical structure with nefiracetam (Fig. 1). Aniracetam is known to potentiate the activity of AMPA-type glu-

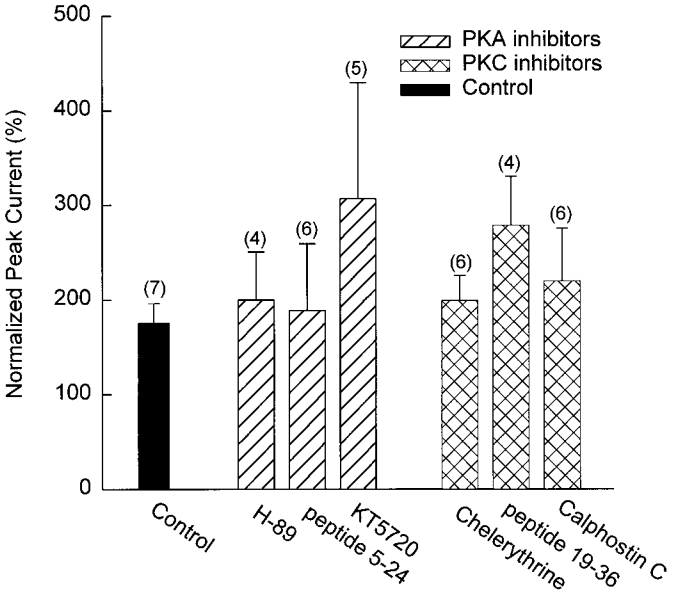


Fig. 9. Summary of the effect of PKA and PKC inhibitors on nefiracetam potentiation of nnAChRs. The average increase in the $\alpha4\beta2$-type currents by 10 nM nefiracetam was not affected by either PKA or PKC inhibitors (mean ± S.D., $n = 4$–7). $P > 0.05$.

tamate receptors at high concentrations of 1 to 5 mM (Ito et al., 1990; Isaacson and Nicoll, 1991; Tang et al., 1991; Partin et al., 1996; Kolta et al., 1998) and the high voltage-activated calcium channels at micromolar concentrations (Yoshii and Watabe, 1994). However, no data are available on aniracetam action on nnAChRs in native brain neurons.

Aniracetam at concentrations of 0.1 nM, 1 nM, 10 nM, and 10 μM markedly and reversibly potentiated $\alpha4\beta2$-type currents in rat cortical neurons (Fig. 12). At a concentration of 0.1 nM, aniracetam consistently potentiated ACh currents (Fig. 12A), indicating that the minimum effective concentration was less than 0.1 nM. Thus, aniracetam was slightly more potent than nefiracetam, which potentiated the currents to a minimum extent at 0.1 nM (Fig. 4C).

Discussion

nnAChR Subunit Dependence of Nootropic Actions. There is general agreement that the nicotinic system plays a major role in higher cognitive functions (Vidal and Changeux, 1996). $\alpha4$, $\beta2$, and $\alpha7$ are the prominent subunits to form the pentameric, heteromeric $\alpha4\beta2$-type or homomeric $\alpha7$-type nnAChRs in the mammalian brain (Whiting and Lindstrom, 1988; Flores et al., 1996). The loss of brain nnAChRs is a neurochemical hallmark of Alzheimer's disease (Vidal and Changeux, 1996; Woodruff-Pak and Hinchliffe, 1997). Compared with the $\alpha4\beta2$ subunits, reductions in the $\alpha7$ subunits seem less extensive in the cortex and hippocampus of Alzheimer's patients (Martin-Ruiz et al., 1999; Burghaus et al., 2000). Thus, $\alpha4\beta2$-type nnAChRs may play an important role in the development of Alzheimer's dementia. In the present study, nefiracetam and aniracetam potently augmented $\alpha4\beta2$-type currents and weakly suppressed $\alpha7$-type currents evoked by ACh in rat frontal cortex neurons. The selective enhancement of $\alpha4\beta2$-type but not $\alpha7$-type currents has implications for understanding the role of nicotinic receptors in this neurodegenerative disorder and in terms of symptomatic and neuroprotective therapy.

Our observations in rat native neurons are different from those in *X. laevis* oocytes expressing the rat $\alpha4\beta2$ or $\alpha7$ nnAChRs. Nishizaki et al. (2000) reported that nefiracetam at 0.1 μM potentiated not only $\alpha4\beta2$ but also $\alpha7$ receptor currents, whereas its analogs aniracetam and piracetam had no effects on these nnAChRs. These discrepancies on the effects of nefiracetam and aniracetam on $\alpha4\beta2$ and $\alpha7$ nnAChRs may be because of the difference in expression systems. The folding, assembly, and subcellular localization of cloned nnAChR subunits are critically dependent upon the nature of host cells (Cooper and Millar, 1997; Sweileh et al., 2000), and the recombinant nnAChRs expressed in *X. laevis* oocytes and several other host cells may not resemble nnAChRs expressed in native mammalian neurons. The properties of nnAChRs can also be influenced by the choice of heterologous expression system (Lewis et al., 1997; Sivilotti et al., 1997). The heterologous expression of $\alpha7$ is regulated not at the transcriptional level, but at the post-translational level, and not all host cell systems seem to express the cellular factors needed for the correct post-translational modifications leading to mature and functional $\alpha7$ AChRs (Aztiria et al., 2000). We also observed that nefiracetam had no po-

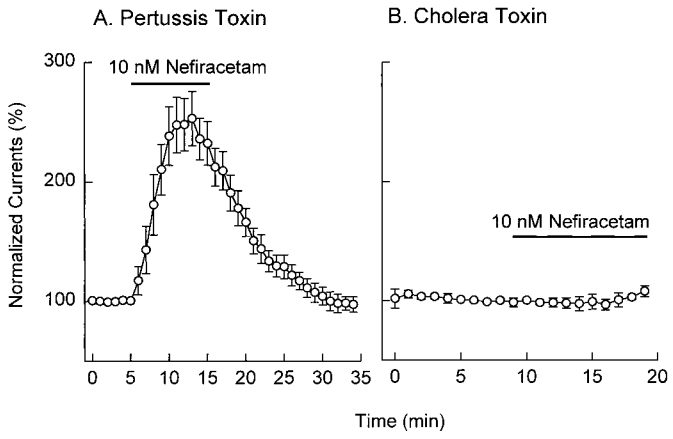


Fig. 10. Cholera toxin, but not pertussis toxin, prevents nefiracetam potentiation in $\alpha4\beta2$-type currents in cortical neurons. The currents were induced by 250 ms 10 μM ACh at 1-min intervals at a holding potential of -70 mV. The neurons were pretreated with 200 ng/ml pertussis toxin, a G_i/G_o inhibitor (A), or 500 ng/ml cholera toxin, a G_s stimulator (B), for 24 to 26 h before recording. Nefiracetam (10 nM) was applied through the external solution in the bath for 10 min. The currents were normalized to the average peak amplitude of the currents recorded in the absence of nefiracetam. $n = 5$ (A) and 4 (B).

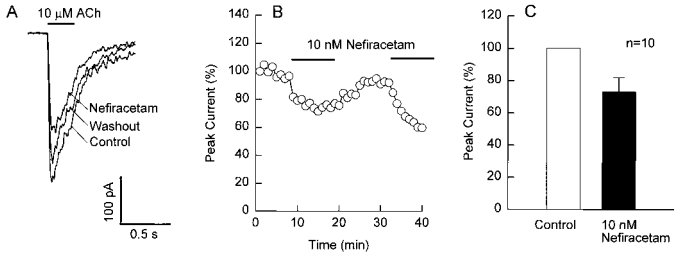


Fig. 11. Nefiracetam does not potentiate but inhibits ACh-induced currents in HEK cells expressing the human $\alpha4\beta2$ nnAChR subunits. A, currents were induced by 10 μM ACh at a holding potential of -70 mV. B, the peak currents were decreased after applying 10 nM nefiracetam in the bath and recovered after washout with drug-free external solution. C, the average of the peak amplitude was decreased 26.9 $\pm$ 8.8% ($n = 10$).

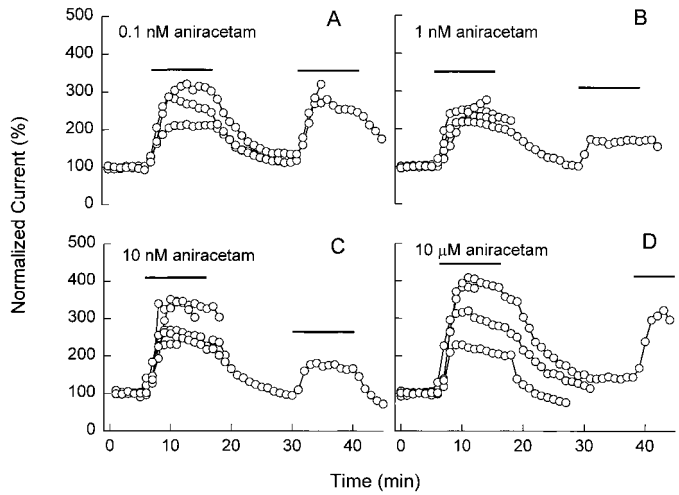


Fig. 12. The potentiation of ACh-induced $\alpha4\beta2$-type currents caused by various concentrations of aniracetam in cortical neurons. Aniracetam was perfused through the bath for 10 min after taking several stable control recordings. Some neurons received second aniracetam treatment after a 25-min washout with the normal external solution. The peak amplitude of current induced by 10 μM ACh at a holding potential of -70 mV was gradually augmented by 0.1 nM (A), 1 nM (B), 10 nM (C), and 10 μM (D) aniracetam. The effect was reversible after washout with aniracetam-free solution.

tentiating effect on cloned human $\alpha4\beta2$ nnAChRs expressed in HEK cells. Therefore, the cloned nnAChRs expressed either transiently in *X. laevis* oocytes or stably in mammalian cell lines may not accurately represent native nnAChRs in neurons. Another factor that complicates the comparison is the sources of nnAChR subunits. We used human $\alpha4\beta2$ subunits expressed in HEK cells, whereas Nishizaki et al. (2000) used rat $\alpha4\beta2$ subunits expressed in *X. laevis* oocytes.

Characteristics of Nefiracetam Potentiation of $\alpha4\beta2$-type Currents. The potentiation of $\alpha4\beta2$-type currents by nefiracetam and the recovery after washout were relatively slow, as has been seen in other studies (Nishizaki et al., 1998; Oyaizu and Narahashi, 1999). The slow potentiation and recovery processes may suggest use-dependent modulation in which the magnitude of effect increases as the receptor is activated by repeated ACh stimulation. However, our study showed that nefiracetam potentiation of $\alpha4\beta2$-type currents was not dependent on the receptor activation. Therefore, the slow onset of enhancement may come from the intracellular signal transduction pathway.

Different from the dual action of nefiracetam on nnAChRs of PC12 cells, in which nefiracetam at 10 μM potentiated the currents evoked by low ACh concentrations and suppressed the currents evoked by high ACh concentrations (Oyaizu and Narahashi, 1999), nefiracetam potentiated $\alpha4\beta2$-type currents induced by all concentrations of ACh tested. It is interesting to note that nefiracetam potentiation was observed even at ACh concentrations that caused saturating responses. The result is similar to ethanol potentiation of $\alpha4\beta2$-type currents (Aistrup et al., 1999). The major effect of nefiracetam on the ACh dose-response relationship could be accounted for by an increase in the maximal response rather than a small shift of ACh dose-response curve. This raises questions regarding the mechanism of nefiracetam potentiation at high ACh concentrations: 1) an increase in single-channel conductance; 2) an increase in channel open probability; 3) a prolongation of open time; 4) the possibility that ACh at high concentrations does not activate all receptors; and 5) a combination of any of the four. Single-channel current recordings are required to elucidate the mechanism underlying this phenomenon.

In *X. laevis* oocytes, short-term treatment with nefiracetam produced a long-lasting potentiation of cloned $\alpha4\beta2$ subunits and *T. californica* nAChRs (Nishizaki et al., 1998, 2000). In contrast, we did not observed long-lasting effects but observed that the enhancement was reversible when nefiracetam was washed away from the cell.

Another characteristics of the nefiracetam-induced potentiation of $\alpha4\beta2$-type currents were the bell-shaped dose-response curve which was also observed in other in vitro studies (Yoshii and Watabe, 1994; Oyaizu and Narahashi, 1999; Nishizaki et al., 1998, 2000). The threshold concentration of nefiracetam for the potentiation of $\alpha4\beta2$-type nnAChRs was about 0.1 nM. In some neurons, nefiracetam at 1 to 10 μM initially potentiated the current to 400% of control, but the current gradually declined to 300% of control. This biphasic effect is also indicative of a bell-shaped dose-response curve observed even in behavioral experiments with nootropic drugs (Nabeshima, 1994; Nabeshima et al., 1994; Hiramatsu et al., 1997). Using *T. californica* nAChRs expressed in *X. laevis* oocytes, nefiracetam at low concentrations (10~100 nM) caused a short-term depression of ACh-induced currents and caused a long-term potentiation at high concentrations (1–10 μM) (Nishizaki et al., 1998). It was proposed that nefiracetam depression was caused by the activation of the pertussis toxin-sensitive, G protein-regulated PKA activity that phosphorylated AChRs. On the contrary, nefiracetam potentiation was caused by the activation of Ca^{2+}-dependent PKC, which also phosphorylated AChRs (Nishizaki et al., 1998). Yoshii et al. (2000) proposed that there were two different sites of action for these nootropics: a higher affinity site responsible for the facilitating action and a low affinity site for the opposite action.

Signal Transduction and Nefiracetam Potentiation. It is well established that protein phosphorylation plays an important role in various neuroreceptors (Huganir and Greengard, 1990; Hoffman et al., 1994). The majority of extracellular signals is mediated by the activation of membrane receptors that are coupled to transducer components located at the inner surface of the plasma membrane. These transducer molecules consist of a family of G proteins, which could modify the activity of nnAChRs indirectly through effector enzymes that produce intracellular signals or directly by a membrane-delimited pathway. Previous studies indicated that nefiracetam modulation of $GABA_A$ receptors (Huang et al., 1996), nAChRs (Nishizaki et al., 1998; 2000; Oyaizu and Narahashi, 1999), and high voltage-gated calcium channels (Yoshii and Watabe, 1994; Yoshii et al., 2000) occurred via protein kinases and G proteins. However, these results are not necessarily consistent. In PC12 cells expressing the $\alpha3$-type nAChRs, the PKA inhibitor and the G_i/G_o protein inhibitor pertussis toxin but not the PKC inhibitor abolished the nefiracetam stimulation of nnAChRs (Oyaizu and Narahashi, 1999). In *X. laevis* oocytes expressing *T. californica* nAChRs or rat nnAChRs ($\alpha4\beta2$ or $\alpha7$), PKC inhibitors but not PKA inhibitors blocked the nefiracetam potentiation of these nAChRs (Nishizaki et al., 1998, 2000). It was proposed that nefiracetam may act on two different signal transduction pathways: one is responsible for pertussis toxin-sensitive G protein-regulated PKA activation and the other for Ca^{2+}-dependent PKC activation. Previous studies also showed that nefiracetam modulated $GABA_A$ receptor currents in rat dorsal root ganglion neurons (Huang et al., 1996) and L-type Ca^{2+} channels in neuroblastoma $\times$ glioma hybrid (NG108–15) cells (Yoshii and Watabe, 1994) by interacting with a PKA pathway.

Our experiments with native cortical neuron nAChRs indicated that both PKA inhibitors (H-89, KT5720, and peptide 5-24) and PKC inhibitors (chelerythrine, calphostin C, and peptide 19-36) did not prevent nefiracetam potentiation. The difference between the data in the literature and our results may be because of the receptor subunit composition, the host cells, and/or the modulators applied.

The activation of G protein-coupled receptors by extracellular agonists promotes interactions between the receptor and the G protein on the interior surface of the membrane. This induces a conversion of GTP to GDP in the G protein α subunit and the dissociation of α subunit from the $\beta\gamma$ heterodimer. Depending on the isoform of the α subunit, the GTP-α subunit complex mediates intracellular signaling either indirectly, by acting on effector molecules such as adenylyl cyclase or phospholipase C, or directly, by regulating ion channel or kinase function (Neer, 1995).

Pertussis toxin and cholera toxin are useful tools to study

the role of G proteins in modulating the function of receptors and ion channels (Zhu and Ikeda, 1994). Pertussis toxin is a selective, irreversible blocker of G_i/G_o. Pertussis toxin catalyzes ADP ribosylation of α-subunits of G_i and G_o proteins, prevents the G_i heterotrimers from interacting with receptors, and blocks their coupling and activation (Kataka et al., 1984). Because the $G_{i\alpha}$ remains in the GDP-bound and inactive state, it becomes unable to inactivate adenylyl cyclase. In contrast, cholera toxin catalyzes ADP-ribosylation of $G_{s\alpha}$, which in turn activates adenylyl cyclase, resulting in an increase in the level of cAMP (Gill and Meren, 1978). cAMP activates cAMP-dependent protein kinases, including PKA.

Although pretreatment with pertussis toxin did not prevent nefiracetam from potentiating $\alpha4\beta2$-type currents, cholera toxin did prevent nefiracetam potentiation. It is possible that the potentiating effect of nefiracetam is not seen because the receptors are already maximally modulated after cholera toxin treatment. However, this possibility was excluded by the following observation. We compared the ACh currents after cholera toxin treatment with those without cholera toxin treatment. These two were not significantly different from each other. After the activation of G_s protein by cholera toxin, there are two major pathways by which $G_{s\alpha}$ proteins could exert their modulating action, one via activation of adenylyl cyclase and the other via membrane-delimited pathway. The lack of effect of PKA and PKC inhibitors on the nefiracetam potentiating action suggests that G_s proteins may regulate the activity of the receptor via membrane-delimited pathways. Another example is the inhibition of the N-type calcium channel by the vasoactive intestinal polypeptide (Zhu and Ikeda, 1994). In this case, the polypeptide inhibition required activation of $G_{s\alpha}$ but was independent of PKA-mediated pathway.

In conclusion, the nootropic drugs nefiracetam and aniracetam potently enhance the $\alpha4\beta2$-type nnAChR response of rat cortical neurons in long-term primary culture. In contrast, nefiracetam and aniracetam slightly inhibit rather than potentiate the $\alpha7$-type currents. The potentiation of $\alpha4\beta2$-type nnAChR can be blocked by a G_s protein modulator but not by a G_i/G_o protein inhibitor or PKA and PKC inhibitors. These results indicate that $\alpha4\beta2$-type nnAChRs are an important site of action of the nootropic nefiracetam and that G_s proteins may play a crucial role in the nefiracetam potentiation.

Acknowledgments

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