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ACCELERATED COMMUNICATION

Nefiracetam Modulates Acetylcholine Receptor Currents via Two Different Signal Transduction Pathways

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ABSTRACT

Nootropic agents are proposed to serve as cognition enhancers. The underlying mechanism, however, is largely unknown. The present study was conducted to assess the intracellular signal transduction pathways mediated by the nootropic nefiracetam in the native and mutant *Torpedo californica* nicotinic acetylcholine (ACh) receptors expressed in *Xenopus laevis* oocytes. Nefiracetam induced a short-term depression of ACh-evoked currents at submicromolar concentrations (0.01–0.1 μM) and a long-term enhancement of the currents at micromolar concentrations (1–10 μM). The depression was caused by activation of pertussis toxin-sensitive, G protein-regulated, cAMP-dependent protein kinase (PKA) with subsequent phosphorylation of the ACh receptors; in contrast, the enhancement was caused by activation of Ca^{2+}-dependent protein kinase C (PKC) and the ensuing PKC phosphorylation of the receptors. Therefore, nefiracetam interacts with PKA and PKC pathways, which may explain a cellular mechanism for the action of cognition-enhancing agents.

Numerous studies have shown that piracetam-like nootropics (or cognition-enhancing agents) can improve various neurotransmissions in the brain, those in the dopaminergic (Funk and Schmidt, 1984), cholinergic (Spignoli and Pepeu, 1987), glutamatergic (Marchi *et al.*, 1990) and γ-aminobutyric acid-ergic (Watabe *et al.*, 1993) systems. Nootropics also facilitate long-term potentiation (Satoh *et al.*, 1986), a model system of memory and learning. These actions are explained by an increase in the release of neurotransmitters from presynaptic terminals (Funk and Schmidt, 1984; Marchi *et al.*, 1990) or an enhancement in neurotransmitter receptor responses at postsynaptic sites (Isaascon and Nicoll, 1991; Ito *et al.*, 1990; Tang *et al.*, 1991). The underlying regulatory mechanism, however, is unknown. We identified the intracellular signal transduction pathways responsible for the nootropic actions by monitoring currents through expressed native and mutant *Torpedo californica* nACh receptors. The results of the present study suggest that nefiracetam is involved in the activation of PKA and PKC, leading to inhibition and potentiation of ACh-evoked currents, respectively.

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Materials and Methods

In vitro transcription and translation in *Xenopus laevis* oocytes

Construction of the plasmids containing the *T. californica* nicotinic ACh receptor subunits has been described previously (Sumikawa and Miledi, 1989). The α, β, γ, δ subunit constructs were transcribed *in vitro* using SP6 RNA polymerase as previously described (Sumikawa and Miledi, 1989). *T. californica* nACh receptors are known to have PKA phosphorylation sites on the γ and δ subunits (Huganir and Greengard, 1990) and PKC phosphorylation sites on the α and δ subunits (Huganir, 1987). The γ and δ subunit mutants that lack PKA phosphorylation sites were constructed using site-directed mutagenesis (Gehle and Sumikawa, 1991); Ser353,354 on the γ subunit and Ser361,362 on the δ subunit were replaced by Ala, and Ser333 on the α subunit and Ser377 on the δ subunit were replaced by Ala for the α and δ subunit mutants that lacked PKC

ABBREVIATIONS: nACh, nicotinic acetylcholine; ACh, acetylcholine; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular free calcium concentration; PTX, pertussis toxin.

phosphorylation sites. After surgical removal from female *X. laevis* frogs and manual separation from the ovary, the isolated oocytes were incubated in Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_2)_2$, 0.41 mM $CaCl_2$, and 7.5 mM Tris, pH 7.6). One day before microinjection, collagenase treatment (0.5 mg/ml) of oocytes was performed. Oocytes were injected (approximately 40 nl) with combinations of normal (α, β, γ, δ) and mutant (mγΔPKA/Ser353,354 mδΔPKA/Ser361, 362) or (mαΔPKC/Ser333 mδΔPKC/Ser377) subunit mRNAs and incubated at 18°.

Two-electrode voltage-clamp recording

The injected oocytes were transferred to the recording chamber 24 to 48 hr after incubation and continuously superfused at room temperature (20 to 22°) in a standard frog Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.0). Ca^{2+}-free extracellular solution consisted of 115 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM HEPES, and 1 mM EGTA, pH 7.0. To remove the effect of the muscarinic ACh receptor, 1 μM atropine was added to the extracellular solution. ACh-activated currents were recorded using two-electrode, voltage-clamp techniques with a GeneClamp-500 amplifier (Axon Instruments, Burlingame, CA) (Nishizaki and Ikeuchi, 1995). The currents were analyzed on a microcomputer using pClamp software (version 6; Axon Instrument). ACh was bath-applied to oocytes. Nefiracetam [DM-9384; *N*-(2,6-dimethylphenyl)-2-(2-oxo-pyrrolidinyl)acetamide] (Daiichi Pharmaceutical, Tokyo, Japan) was dissolved in distilled water at 1 mM for stock solution and diluted into concentrations required with the extracellular solution.

Assay of $[Ca^{2+}]_i$

Oocytes were injected with Calcium Green-1 (15 μM final concentration; Molecular Probes, Eugene, OR) and were incubated at 18° for 30 min. The oocytes were transferred to the recording chamber onto the stage of a Nikon DIAPHOT 300 microscope and were bathed at room temperature (20–22°) in standard or Ca^{2+}-free frog Ringer's solution superfused continuously. The oocytes were viewed with a ×4 UV fluor Nikon objective lens and the images were acquired at 2-sec intervals with a xenon confocal laser-scanning microscope (Nikon Xenon Power Supply XPS-100; Nikon, Tokyo, Japan) attached to an intensified charge-coupled device camera (ARGUS-50/CA; Hamamatsu Photonics, Japan). The Calcium Green signal was long pass-filtered (490 nm). Images were analyzed with ARGUS-50/CA software (version 3.0). To compensate different levels of dye loading between oocytes, ΔI (intensity of Ca^{2+} signal after application of ACh − basal intensity) and the ratio of the ΔI to the basal intensity were calculated; to compensate different levels of functional receptor expression, ACh-evoked currents were recorded in Ca^{2+}-free extracellular solution (ACh-gated channel currents). Ca^{2+} mobilizations therefore were normalized by the ΔI/basal intensity/ACh-gated channel current.

Results and Discussion

In our *X. laevis* oocyte expression system for the wild-type nACh receptor, 100 μM ACh evoked inward membrane currents (Fig. 1A). Currents were recorded at 10-min intervals in which spontaneous attenuation of the currents was within 5% during experiments (data not shown). Lower (submicromolar) concentrations of the nootropic nefiracetam reduced ACh-evoked currents to 30 ± 9% (0.01 μM) and 38 ± 11% (0.1 μM) of control after a 10-min treatment (Fig. 1A). The inhibitory effect was slowly recovered; the currents reached the control level (104 ± 19 and 103 ± 20% for 0.01 and 0.1 μM nefiracetam, respectively) 70 min after treatment (Fig. 1A). In contrast, higher (micromolar) concentrations of nefiracetam enhanced the currents in a time-dependent manner

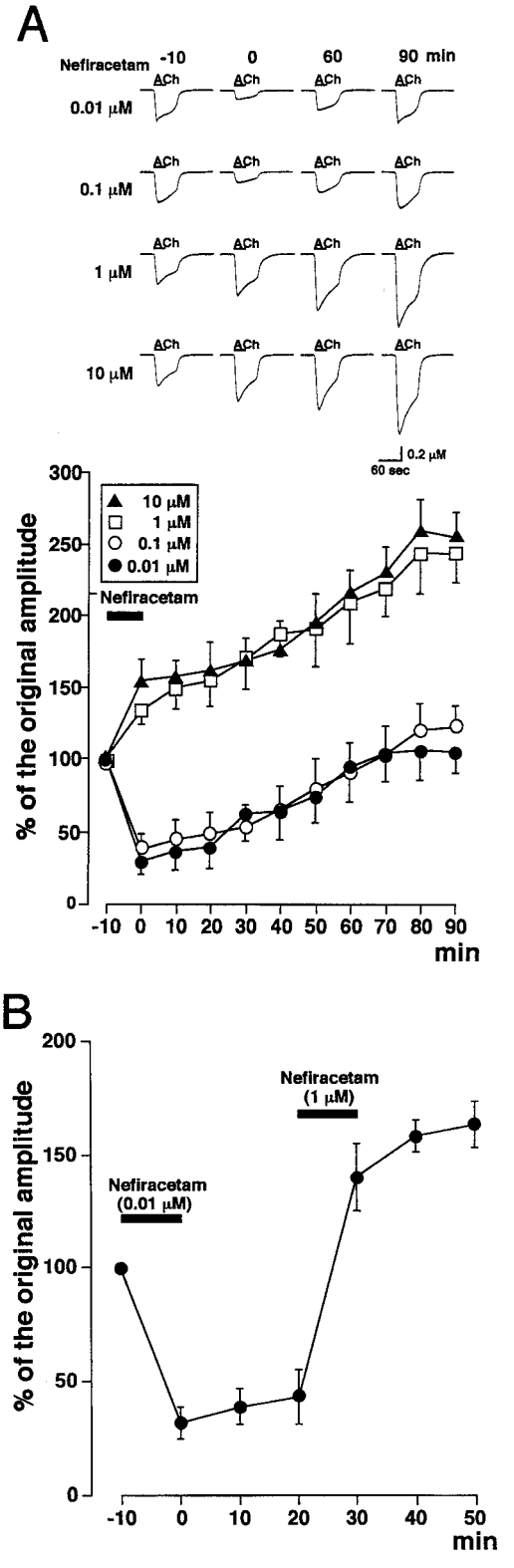

Fig. 1. Effects of nefiracetam on ACh-evoked currents. A, 100 μM ACh was applied to a single oocyte expressing the normal ACh receptors at 10-min intervals. The oocyte was treated with nefiracetam at 0.01, 0.1, 1 and 10 μM for 10 min. The illustrated currents were recorded at the times indicated. The holding potential was −30 mV. Inward currents correspond to inward deflections. The time-course effects of nefiracetam on the currents are summarized in lower column. *Points,* mean percent ± standard deviation of the original amplitude (−10 min) from seven oocytes. B, An oocyte was treated with 0.01 μM followed by 1 μM nefiracetam (seven oocytes).

during treatment and continued to do so after washing-out of the drug (Fig. 1A). The current potentiating effect was long-lasting, the currents reaching 243 ± 20 and 255 ± 18% at 1 and 10 μM, respectively, 90 min after treatment (Fig. 1A). These results indicates that nefiracetam exerted dose-dependent biphasic effects on ACh-evoked currents: a short-term depression at submicromolar concentrations and a long-term enhancement at micromolar concentrations. When 1 μM nefiracetam was applied in the process of the current suppression by 0.01 μM nefiracetam, the drug action was switched from suppression to enhancement (Fig. 1B). This suggests that at least two different signal transduction pathways were involved in such a biphasic action of nefiracetam.

T. californica nACh receptors form nonselective cation channels; ACh-evoked currents in *X. laevis* oocytes that express the ACh receptors are composed of ACh-gated channel currents and Ca^{2+}-dependent chloride currents that are evoked by Ca^{2+} entry through the ACh receptor channels (Miledi and Parker, 1984). To ascertain the site of the nefiracetam action, Ca^{2+}-sensitive chloride currents were induced by activation of the intrinsic muscarinic ACh receptors. Nefiracetam had no effect on the Cl^- currents (Fig. 2A), which suggests that nefiracetam modulated ACh-gated channel currents but not chloride currents. To obtain further evidence for this, $[Ca^{2+}]_i$ was assayed. ACh increased $[Ca^{2+}]_i$ in the presence of Ca^{2+}-containing extracellular solution, whereas no increase was observed in Ca^{2+}-free extracellular solution (Fig. 2B), which indicated that the ACh receptor channels permeated calcium and that the source of the $[Ca^{2+}]_i$ increase was extracellular. Nefiracetam, however, did not induce further rise in $[Ca^{2+}]_i$, although it potentiated ACh-evoked currents (Fig. 2B), which implies that nefiracetam did not affect Ca^{2+} permeability; in other words, nefiracetam potentiated ACh-evoked currents by modulating ACh receptor currents, possibly Na^+ currents, but not by the secondary effect of Ca^{2+}-sensitive chloride currents as a consequence of intracellular Ca^{2+} rise.

Subsequently, an attempt was made to assess the intracellular signals that mediate the nefiracetam-induced current depression and potentiation. H-89, a selective inhibitor of PKA, blocked the inhibitory action of nefiracetam at submicromolar concentrations on ACh-evoked currents; however, it potentiated the currents to 172 ± 19% of control 30 min after treatment with 0.01 μM nefiracetam (Fig. 3A), which suggests that nefiracetam at lower concentrations reduced the currents via PKA activation. ACh-evoked currents were inhibited drastically in the presence of the PKA activator forskolin (21 ± 8% of control) (Fig. 3A), providing indirect evidence that nefiracetam interacts with a PKA pathway, leading to inhibition of the currents. Nefiracetam (0.01 μM) enhanced ACh receptor currents in oocytes treated with PTX, a G protein ($G_{i/o}$) inhibitor, to the same level as observed in H-89 (Fig. 3A), which suggests that the inhibitory action of nefiracetam was mediated by PKA under the regulation of PTX-sensitive G proteins. In addition, the facilitatory action of 1 μM nefiracetam was more enhanced in the presence of H-89, the currents reaching 236 ± 18% (five oocytes) 30 min after treatment (data not shown). This suggests strongly that higher concentrations of nefiracetam can still activate PKA and decrease the currents, although such inhibitory effects will be masked by the apparent current potentiation.

To further examine whether the current depression by

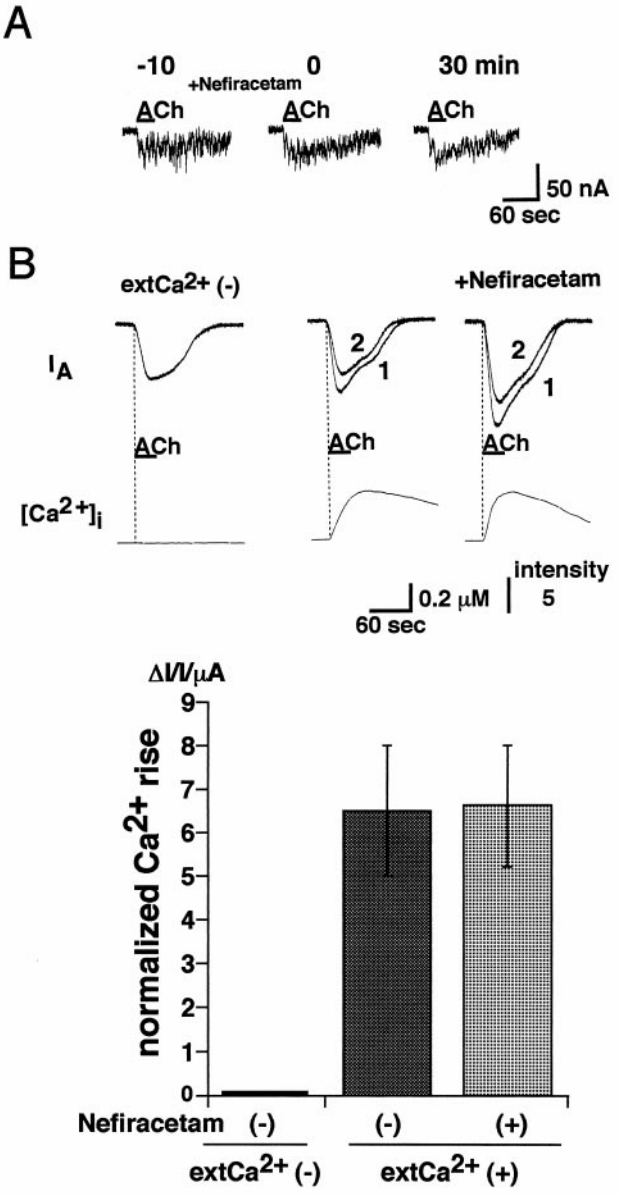

Fig. 2. Effect of nefiracetam on Ca^{2+}-dependent chloride currents and Ca^{2+} influx through the normal nicotinic ACh receptors. A, 100 μM ACh was applied to an oocyte without expression of the ACh receptors in atropine-free frog Ringer's solution. The evoked Cl^- currents were recorded 10 min before, and 0 and 30 min after treatment with nefiracetam (1 μM). The holding potential was −30 mV. Inward currents correspond to inward deflections. B, Two-electrode voltage-clamp was made to a Calcium Green-loaded oocyte expressing the normal ACh receptors. Intracellular Ca^{2+} mobilizations were monitored in the presence and absence of nefiracetam (1 μM). Simultaneously, 100 μM ACh-evoked currents (I_A) were recorded in Ca^{2+}-containing (*1*) and Ca^{2+}-free extracellular solution (*ext*Ca^{2+} *(−)*; *2*). The holding potential was −30 mV. Inward currents correspond to inward deflections. The Ca^{2+} rise was normalized by Δ increase of intensity/basal intensity/current amplitude (*2*). Data are presented as the mean ± standard deviation of seven independent experiments.

nefiracetam is caused by PKA phosphorylation of the receptors, mutant ACh receptors lacking potent PKA phosphorylation sites on the γ and δ subunits (m$\gamma\Delta$PKA/Ser353, 354 m$\delta\Delta$PKA/Ser361, 362) were expressed in oocytes. There, nefiracetam (0.01 μM) did not decrease ACh-evoked currents in the mutant ACh receptors; conversely, it increased them to 159 ± 20% of control 30 min after treatment (Fig. 3A). These results indicate that nefiracetam inhibited ACh receptor cur-

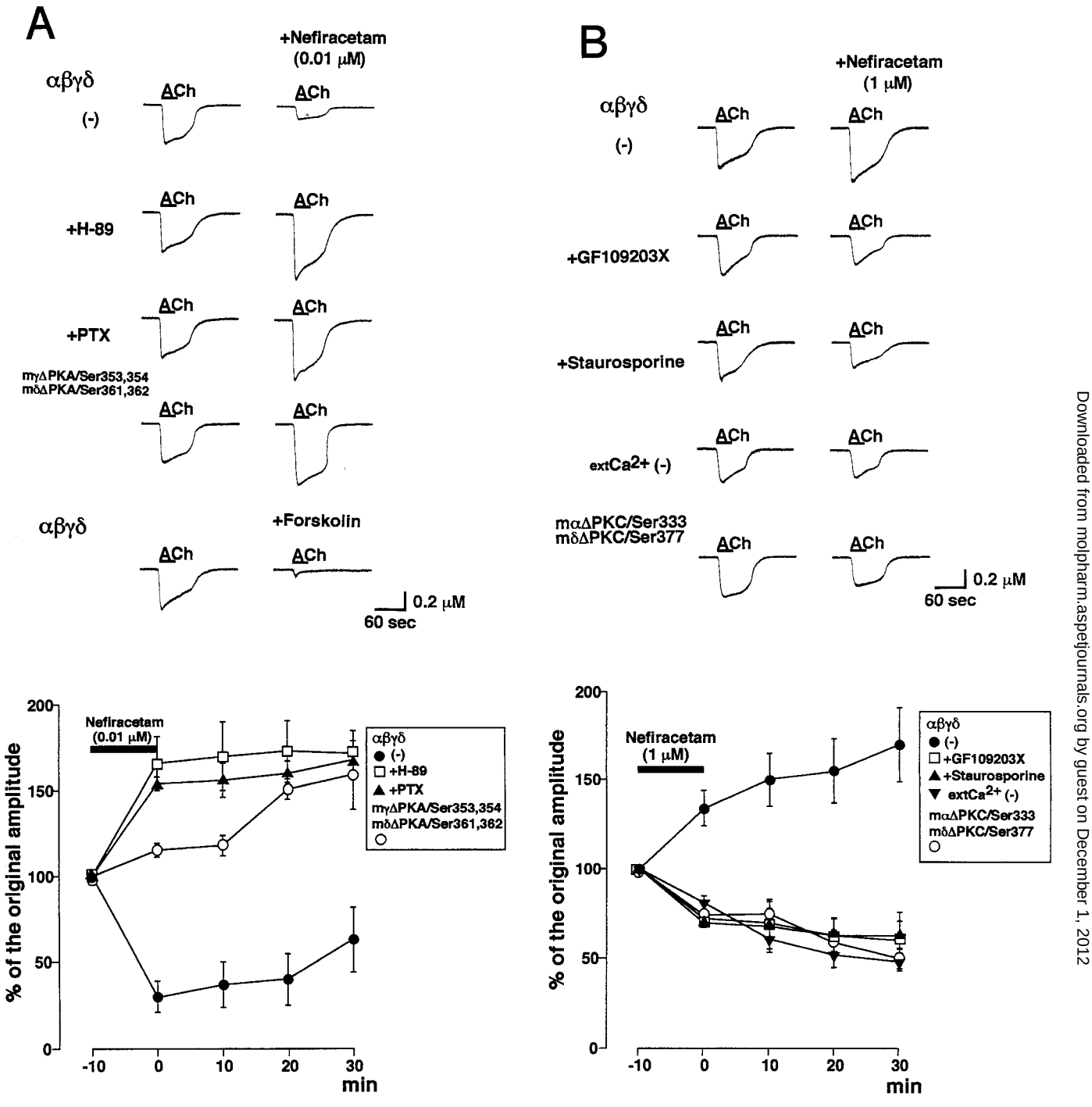

Fig. 3. Regulation of ACh-evoked currents by nefiracetam-mediated PKA and PKC activation. A, 100 μM ACh was applied to an oocyte expressing the normal or mutant ACh receptors lacking PKA phosphorylation sites in the presence and absence of H-89 (1 μM). Some oocytes were treated with 0.1 μg/ml PTX for 24 hr before experiments. The illustrated currents were recorded 10 min before and 30 min after treatment with 0.01 μM nefiracetam. In other experiments, ACh was applied to a single oocyte expressing the normal ACh receptors before and after 15 min treatment with forskolin (20 μM) (five oocytes). The holding potential was −30 mV. Inward currents correspond to inward deflections. *Points*, mean percent ± standard deviation of the original amplitude (−10 min) from seven oocytes; *bars*, standard deviation. B, 100 μM ACh was applied to an oocyte expressing the normal or mutant ACh receptors lacking PKC phosphorylation sites in the presence and absence of 100 nM GF109203X, 5 μM staurosporine, or in Ca^{2+}-free extracellular solution. The illustrated currents were recorded 10 min before and 30 min after treatment with nefiracetam (1 μM). The holding potential was −30 mV. Inward currents correspond to inward deflections. *Points,* mean percent ± standard deviation of the original amplitude (−10 min) from seven oocytes.

rents by PKA activation and the subsequent PKA phosphorylation of the receptors.

In an earlier study using NG108–15 cells, nefiracetam enhanced the activity of neuronal L-type calcium channels in a fashion that mimics the effect of dibutyryl cAMP, and the enhancement was inhibited by PTX (Yoshii and Watabe, 1994), supporting the idea that the action of nefiracetam is associated with PTX-sensitive G proteins and their regula-

tion of PKA activation. No evidence, however, has been provided for the mechanism by which PTX-sensitive G proteins ($G_{i/o}$-proteins) can facilitate adenylyl cyclase, not even for any well characterized receptor site as a target of nootropics (Gouliaev and Senning, 1994). How, then, could nefiracetam activate PKA? It might stimulate unknown cytosolic PTX-sensitive G proteins involving PKA activation.

On the other hand, the selective PKC inhibitor, GF109203X (Heikkila *et al.*, 1993) or staurosporine inhibited potentiation of ACh-evoked currents by 1 μM nefiracetam: the currents reaching 60 $\pm$ 16 or 63 $\pm$ 12% of control at 30 min washing (Fig. 3B). This suggests that nefiracetam potentiated ACh receptor currents via PKC activation. The potentiation was not observed in Ca^{2+}-free media; instead, the currents were reduced to the same extent as achieved in the presence of the PKC inhibitors (Fig. 3B). The finding that ACh never increased $[Ca^{2+}]_i$ in Ca^{2+}-free media (Fig. 2B) suggests that nefiracetam interacted with a Ca^{2+}-dependent PKC pathway. Furthermore, 1 μM nefiracetam exhibited no current potentiation in the mutant ACh receptors that lacked potent PKC phosphorylation sites on the α and δ subunits (m$\alpha$$\Delta$PKC/Ser333m$\delta$$\Delta$PKC/Ser377) (Fig. 3B). These results indicate that nefiracetam potentiated ACh-gated channel currents by activation of Ca^{2+}-dependent PKC and the subsequent PKC phosphorylation of the receptors. Nefiracetam, therefore, seems to act on two different signal transduction pathways; one is responsible for PTX-sensitive G protein-regulated PKA activation and the other for Ca^{2+}-dependent PKC activation.

At present, the type of PKC pathways with which nefiracetam interacts remains unknown. Of PKCs discovered, only the conventional PKC (cPKC) isozymes (α, βI, βII, γ) are activated in the presence of Ca^{2+} and diacylglycerol after the activation of phospholipase C (Exon, 1994; Liscovitch and Cantley, 1994). There is evidence for a significant role of *cis*-unsaturated free fatty acids in sustained PKC activation, possibly by binding to the C1 domain of PKC (Nishizuka, 1995). It is also suggested that the nACh receptor is capable of activating phospholipase C-mediated PKC (Eusebi *et al.*, 1987). Taken together, nefiracetam may sustain nACh receptor-mediated cPKC activation as *cis*-unsaturated free fatty acids do, leading in turn to a prolonged potentiation of ACh-evoked currents as a result of PKC phosphorylation of the receptors.

Previous studies have shown that nefiracetam modulates γ-aminobutyric $acid_A$ receptor currents (Huang *et al.*, 1996) or L-type calcium channel-operated currents (Yoshii and Watabe, 1994) by interacting with a PKA pathway. In addition to a PKA pathway, the present study identified a nefiracetam-mediated PKC pathway. Lines of evidence suggest that nootropic agents serve as cognition enhancers by facilitating a variety of neurotransmissions, including cholinergic systems (Funk and Schmidt, 1984; Spignoli and Pepeu, 1987; Marchi *et al.*, 1990; Watabe *et al.*, 1993). The results presented here demonstrate that the nootropic nefiracetam can influence two signal transduction pathways linked to PKA and PKC activation. This may explain that nootropics potentially have multiple downstream targets, providing a clue to understand the cellular mechanism for modification of various synaptic transmissions.

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