Identification and Pharmacological Profile of a New Class of Selective Nicotinic Acetylcholine Receptor Potentiators


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ABSTRACT

Here we report the discovery, by high-throughput screening, of three novel (2-amino-5-keto)thiazole compounds that act as selective potentiators of nicotinic acetylcholine receptors. Compound selectivity was assessed at seven human nicotinic acetylcholine receptors (α1β1γδ, α2β4, α3β2, α3β4, α4β2, α4β4, and α7) expressed in mammalian cells or Xenopus oocytes. At α2β4, α4β2, α4β4, and α7, but not α1β1γδ, α3β2, or α3β4, submaximal responses to nicotinic agonists were potentiated in a concentration-dependent manner by all compounds. At similar concentrations, no potentiation of 5-hydroxytryptamine, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, GABA_A, and N-methyl-D-aspartate receptors or voltage-gated Na^+ and Ca^2+ channels was observed. Furthermore, these compounds did not inhibit acetylcholine esterase. Further profiling revealed that these compounds enhanced the potency and maximal efficacy of a range of nicotinic agonists at α4β2 nicotinic acetylcholine receptors, a profile typical of allosteric potentiators. At concentrations required for potentiation, the compounds did not displace [3H]epibatidine from the agonist-binding site, and potentiation was observed at all agonist concentrations, suggesting a noncompetitive mechanism of action. Blockade of common second messenger systems did not affect potentiation. At concentrations higher then required for potentiation the compounds also displayed intrinsic agonist activity, which was blocked by competitive and noncompetitive nicotinic acetylcholine receptor (nAChR) antagonists. These novel selective nicotinic receptor potentiators should help in clarifying the potential therapeutic utility of selective nAChR modulation for the treatment of central nervous system disorders.

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels formed by the assembly of five subunits. Each subunit is composed of a large extracellular N terminus, four transmembrane regions, and a short extracellular C terminus (Lindstrom, 2000). The orthosteric agonist-binding site is located in the N terminus, at the interface between two adjacent subunits. Receptor activity is also subject to both positive and negative modulation at allosteric sites by steroids, bivalent cations, alcohols, and a range of drugs (Pereira et al., 2002).

Seventeen distinct nAChR subunits have been identified. Nicotinic receptors of the neuromuscular junction are composed of α1, β1, δ, and γ or ε, with a stoichiometry of α2β2γεδ, whereas neuronal nAChRs are composed of either heteromeric (α2–6 and β2–4) or homomeric (α7–10) subunit combinations. The subunit composition and stoichiometry of native neuronal nAChRs is not fully defined, although peripheral ganglionic nAChRs contain the α3 subunits and nAChRs with α4β2 or α7 subunits are broadly expressed in the central nervous system (McGehee and Role, 1995).

There is currently significant interest in the development of selective nAChR agonists and positive allosteric modulators for the treatment of various neurological and psychiatric disorders (Lloyd and Williams, 2000;Astles et al., 2002). This interest stems from several lines of evidence implicating...
nAChRs in both physiological and pathological mechanisms in the central nervous system. 

α7 and α4β2 nAChRs are normally expressed at high levels in areas involved in learning and memory and play a physiological role in the modulation of neurotransmission within these regions (Dani, 2001; Sher et al., 2004). Under pathological conditions, such as in Parkinson’s and Alzheimer’s disease or schizophrenia, reduced cholinergic activity and loss or malfunction of nAChRs are common features and can be correlated to cognitive deficits and progressive dementia (Leonard et al., 2000; Court et al., 2001; Quik and Kulak, 2002). The involvement of nAChRs in the modulation of cognitive function has been demonstrated in both animal models and clinical studies (Levin and Rezvani, 2000). In animal models, improvements in memory performance can be obtained using nonselective nAChR agonists, such as nicotine and epibatidine, whereas nAChR antagonists such as mecamylamine and dihydro-β-erythroidine induce learning impairments. In clinical studies, broad nAChR agonists are similarly reported to improve attention, memory, and learning in both normal and disease states (Levin and Rezvani, 2000). The therapeutic utility of nonselective nAChR agonists, however, is limited by a low therapeutic index, mainly caused by activation of the autonomic nervous system, and, possibly, muscle receptors. The expectation that selective central nAChR agonists may show a better side effect profile, while retaining efficacy, is supported by the recent development of subtype selective nAChR agonists, which confirmed a role for α7 and α4β2 nAChR subtypes in the modulation of cognitive function in animal models (Lippiello et al., 1996; Meyer et al., 1997; Gatto et al., 2004).

A more physiological approach for targeting central nAChRs is through the use of positive allosteric modulators, which would potentiate the receptor only when the endogenous cholinergic system is operating and would not chronically stimulate the receptors themselves. However, selective nAChR potentiators are only recently starting to emerge (Hurst et al., 2005; Sher et al., 2005), and the nonselective potentiators currently available are not suitable for examining their therapeutic value in animal models or clinical studies. For example, 5-hydroxyindole has been shown to selectively potentiate α7 nAChRs compared with other nAChR subtypes (Zwart et al., 2002) but is active only at high micromolar to millimolar concentrations and displays cross-reactivity at 5-HT₃Rs (Kooymann et al., 1993). Ivermectin displays selectivity for α7 over other nAChR subtypes (Krause et al., 1998) but also acts on glycine (Shan et al., 2001) and purinergic 2X receptors (Khakh et al., 1999). Interestingly, galantamine, an acetylcholine (ACh) esterase inhibitor used for the treatment of Alzheimer’s disease, is reported to act also as a positive allosteric modulator of nAChRs, and this activity might underlie part of its clinical efficacy (Maelicke et al., 2000; Woodruff-Pak et al., 2002). However, galantamine is not selective across the nAChR subtypes, it also modulates NMDA receptors (Moriguchi et al., 2004), and it is an ACh esterase inhibitor.

We recently performed a high-throughput screen of a large Eli Lilly proprietary chemical library on recombinant human nAChRs, using a calcium dye and the fluorescent imaging plate reader (FLIPR) technology. The screening protocols were designed to allow for the identification of both agonist and antagonists and potentiators. The present study reports on the identification of a novel chemical class of selective nicotinic receptor modulators that potentiate central but not ganglionic or neuromuscular nAChRs.

### Materials and Methods

**nAChR Expression in Xenopus Oocytes and Electrophysiological Characterization.** Adult female *Xenopus laevis* frogs were obtained from Blades Biological (Edendbridge, UK) and housed at Lilly, Windlesham (Surrey, UK), according to Home Office regulations.

All experimental protocols have been detailed previously (Zwart et al., 2002). In brief, nuclei of stages V and VI oocytes were injected with plasmds containing human α7, α4, α3, β2, or β4 nicotinic receptor subunits (in pcDNA3) using a Drummond variable volume microinjector (Drummond Scientific, Broomall, PA). Approximately 2 ng of α7, or a combination of 1 ng of α4 or α3 and 1 ng of β2 or β4, was injected into each oocyte in a total injection volume of 18.4 nl. After CDNA injection, oocytes were incubated for 3 to 5 days at 18°C in a modified Barth’s solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 15 mM HEPES, and 50 mg/l noremycin, pH 7.6, 235 mOsm] before experiments.

During experiments, oocytes were placed in a recording chamber and continuously superfused with saline (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.3, 235 mOsm), at a rate of ~10 mI/min. A BP5-8 solution exchange system (ALA Scientific Inc., Westbury, NY) was used to switch between control and drug-containing saline. Oocytes were superfused with drug-free solution for 5 min (2 min for α7) between agonist applications to allow the nAChRs to recover from desensitization. Single oocytes were used for each concentration of potentiator tested because effects of potentiation were slow to reverse. All experiments were performed at room temperature.

Currents were recorded from oocytes impaled by two microelectrodes filled with 3 M KCl (0.5–2.5 Mf), and a holding potential of ~60 mV was applied, using a Geneclamp 500B amplifier (Axon Instruments, Foster City, CA). Membrane currents were filtered (four-pole low-pass Bessel filter, ~3 dB at 1 kHz), digitized (1000 samples/record), and stored on disk for off-line computer analysis.

**Mammalian Cell Culture.** All cells were grown at 37°C in a humidified incubator with 95% air and 5% CO₂. HEK-293 cells stably expressing human recombinant α2β4, α3β2, α4β2, α4β4, or α4β4-human nAChRs, chimera human α7/mouse 5-HT₃ receptors, or human 5-HT₃ receptors were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, and 50 μg/ml gentamicin. Rhabdomysarcoma cells were cultured in the same medium as detailed above, without geneticin. GH4 cells stably expressing human recombinant α7 receptors were cultured in F-10 nutrient mixture supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine, and 50 μg/ml gentamicin.

For FLIPR experiments, all cell lines, except α3β2 nAChRs, were plated at 0.5 or 1.0 x 10⁴ cells/ml (100 μl/well) into poly-d-lysine-coated 96-well plates and incubated overnight, at 37°C, before assaying. HEK cells expressing α3β2 nAChRs, however, were plated 48 h before use and incubated at 29°C to aid functional receptor expression.

For the preparation of primary cultures of cortical neurons, used for the study of native AMPA, GABAₐ, and NMDA receptors and voltage-gated Ca²⁺ and Na⁺ channels, pregnant Sprague-Dawley rats (Harlan, Bicester, Oxford, UK) were anesthetized in a CO₂ atmosphere and subsequently killed by dislocation of the neck, according to standard licensed procedures. Cortices of the 17- to 18-day-old fetuses were dissected, trypsinized, and triturated, and the isolated cells were plated onto poly-d-lysine-coated 96-well microtiter plates.
(BioCoat; BD Biosciences, San Jose, CA) at a density of 6.6 × 10^5 cells/ml, in a volume of 0.1 ml of neurobasal medium containing B27 supplement (GIBCO BRL, Gaithersburg, MD) and incubated for 7 to 9 days before use.

**Cell Population Calcium Measurements Using a FLIPR.** Experimental protocols have been detailed previously (Craig et al., 2004). In brief, cells in 96-well plates were loaded with 10 μM Fluo-3-AM/0.05% Pluronic F-127 in Tyrode’s buffer (137 mM NaCl, 2.7 mM KCl, 2.5 mM CaCl_2, 1 mM MgCl_2, 12 mM NaHCO_3, 0.2 mM NaHPO_4, and 5.5 mM glucose, pH 7.3) by incubation at room temperature in the dark. After 1 h, media were replaced with Tyrode’s buffer in the absence of Fluo-3, and cells were transferred to a FLIPR (Molecular Devices, Sunnyvale, CA) for experiments. Baseline fluorescence was recorded every 1 s for 60 s, and drug addition was made after 10 s to allow an initial baseline to be viewed. Once added, drugs were present for the duration of the experiments. Parameters for drug addition to the cell plate were programmed using FLIPR software, and compound delivery was automated through a 96-tip head pipetter. Responses were measured as peak fluorescence minus basal fluorescence intensity and are expressed as a percent.

**Nicotinic Receptor Radioligand Binding Scintillation Proximity Assay.** Ninety-six-well SPA radioligand binding assays were performed in a final volume of 250 μl of Tris buffer with 1 mM MgCl_2, 5 mM KCl, 2.5 mM CaCl_2, 1 mM MgCl_2, 12 mM NaHCO_3, 0.2 mM NaHPO_4, and 5.5 mM glucose, pH 7.3). The homogenate was centrifuged twice (40,000 g) and resuspended homogenate was assayed in a scintillation proximity assay (SPA) beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). A HEK cell line stably expressing this human 5-HT_3 receptor cDNA was developed after transfection of HEK cells using GenePorter (Gene Therapy Systems Inc., San Diego, CA) and growth under genetic resistance selection (500 μg/ml). Clones were isolated under limiting dilution, and a final clone was selected by screening for function using a FLIPR.

2087101, 2087133, and TC-2559 were synthesized at Lilly (Erl Wood, UK). ACh, nicotine, and mecamylamine were purchased from Sigma-RBI (Poole, UK). Epibatidine, cytisine, galantamine, dihydrro-β-erythroidine (dihE), okadaic acid, and calyculin A were from Tocris Cookson (Bristol, UK). Phorbol myristate acetate (PMA), H-7, staurosporine, cholin, pertussis toxin, and forskolin were from Calbiochem (San Diego, CA). Fluo-3-AM and Pluronic F-127 were from Molecular Probes (Leiden, The Netherlands). All cell culture reagents were from Sigma (Poole, Dorset, UK), except for fetal calf serum, which was from Invitrogen.

**Materials.** All expression constructs containing human nicotinic receptor subunit cDNA in pCfDNA3 and stable cell lines expressing nAChRs were obtained from Merck Research Laboratories (La Jolla, CA). Chimeric human α7/mouse 5-HT_3 nicotinic receptors and the HEK α7/5-HT_3 receptor cell line were constructed as detailed previously (Craig et al., 2004). The complete coding sequence for the human 5-HT_3 receptor was amplified from a human hippocampus cDNA library (QUICK-Clone; BD Biosciences) using PCR. A 1519-bp fragment was isolated using primers designed to anneal to loci within the 5’- and 3’-untranslated regions of the 5-HT_3 sequence (GenBank accession no. D49394; forward primer, ACTCTTATGCT-TGAAAGCTC; reverse primer, AAATCTCTGTGCCCACTTAA). This initial product was used as a template in a further round of PCR made with nested primers that were modified to introduce BamHI and XhoI restriction sites at the 5’ and 3’ ends of the product, respectively (forward primer, TAAATGCTTCTGATCTCGT-GGGTCC; reverse primer, CAATCTGGACTAAGCAGTGTAC-CCCT). For expression studies, the product of the second PCR was ligated into the multiple cloning sequence of pCDNA3.1 (+) (Invitrogen, Paisley, UK) at the BamHI and XhoI sites. The sequence of the construct was verified by automated sequencing (Cytomyx, Cambridge, UK). A HEK cell line stably expressing this human 5-HT_3 receptor cDNA was developed after transfection of HEK cells using GenePorter (Gene Therapy Systems Inc., San Diego, CA) and growth under genetic resistance selection (500 μg/ml). Clones were isolated under limiting dilution, and a final clone was selected by screening for function using a FLIPR.

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**Results.**

**Potentiation of α2β4, α4β2, α4β4, and α7 nAChRs Stably Expressed in Mammalian Cells by 2087101, 1078733, and 2087133.** The activity of three novel (2-amino-5-keto)thiazole compounds 2087101, 1078733, and 2087133 (Fig. 1) was assessed. Over the concentration range tested (1 nM-30 μM), each of the compounds caused a concentration-dependent potentiation of responses mediated by α2β4, α4β2, α4β4, and α7 but not α1β1γ, α3β4, or α3β2 nAChRs (Fig. 2B). α2β4 nAChRs were potentiated to a maximum of 221 ± 8, 170 ± 6, and 145 ± 4% of low epibatidine controls in the presence of 2087101, 2087133, and 1078733, respectively. The same values for α4β4 were 312 ± 6, 293 ± 8, and 200 ± 4%; for α4β2, 289 ± 12, 407 ± 20, and 350 ± %; and for α7, 275 ± 18, 227 ± 18, and 324 ± 15%.

For α2β4, α4β2, and α4β4 nAChRs, maximal potentiation of epibatidine-induced responses was observed after 10 min

previously (Ellman et al., 1961) and human recombinant acetylcholinesterase expressed in HEK-293 cells.
of preincubation with 3 μM 2087101, 1078733, and 2087133, whereas at higher concentrations, an inhibitory effect was observed; the latter was also apparent for α3β4 and α3β2 nAChRs (Fig. 2B).

**Potentiation of α4β2, α4β4, and α7 nAChRs Expressed in Xenopus Oocytes by 2087101, 1078733, and 2087133.** The activity of compounds 2087101, 1078733, and 2087133 was confirmed using voltage-clamped Xenopus oocytes expressing exogenous human α4β2, α7, α4β4, α3β4, and α3β2 nAChRs. The effect of 3 μM 2087101, 1078733, and 2087133 on submaximal (EC_{10-30}) ACh-induced inward current responses was assessed after coapplication (heteromeric nAChRs) or a 2-min preincubation (α7 nAChRs), as indicated (Fig. 3A). The amplitudes of submaximal ACh-induced responses (100%) at α4β2, α4β4, and α7 nAChRs were potentiated to 742 ± 63, 255 ± 32, and 369 ± 149% in the presence of 3 μM 2087101; 1274 ± 60, 695 ± 182, and 126 ± 28% in the presence of 3 μM 2087133; and 1939 ± 269, 853 ± 220, and 244 ± 16% in the presence of 3 μM 1078733, respectively. No potentiation of ACh-induced responses was observed in the presence of 2087101 (96 ± 9 and 89 ± 18%), 2087133 (96 ± 4 and 105 ± 3%), or 1078733 (87 ± 20 and 90 ± 7%) at α3β2 and α3β4 nAChRs, respectively, compared with controls.

Submaximal ACh-induced responses at α4β2, α4β4, and α7 nAChRs were potentiated in a concentration-dependent manner by 2087101 (between 30 nM and 30 μM), yielding EC_{50} values for potentiation of 0.99 and 1.1 μM at α4β2 and α4β4 nAChRs, respectively (Fig. 3B). The data were not robust enough to allow for a sigmoidal fit of the α7 results. Inhibition of ACh-induced currents at high concentrations of potentiator (10–30 μM) was seen in the oocyte system, similar to the FLIPR results described above.

**Effects of 2087101 on the Potency and Efficacy of Nicotinic Agonist-Induced nAChR Responses.** Using 2087101 as an example of this novel class of potentiator, studies were undertaken to determine whether these compounds increased the apparent affinity and efficacy of nAChRs for both full and partial agonists.

Firstly, potentiation was assessed as a function of epibatidine concentration at α3β4, α4β2, α4β4, and α7 nAChRs stably expressed in mammalian cells. The effect of 1 to 3 μM 2087101 on a range of epibatidine-induced responses (0.1 nM to 10 μM) was assessed after an ~10-min preincubation with potentiator. 2087101 caused a significant increase in both the potency and efficacy of epibatidine at α4β2, α4β4, and α7 nAChRs, whereas it had no discernible effect on epibatidine-induced responses at α3β4 nAChRs (Fig. 4A; Table 1).
In Xenopus oocytes expressing α4β2 nAChRs, the effect of coapplication of 1 μM 2087101 on a range of ACh-induced inward current responses (100 nM to 1 mM) was assessed and compared with the responses in the absence of potentiator (Fig. 4B). 2087101 caused an increase in both the potency and efficacy of acetylcholine. In control oocytes, acetylcholine displayed EC50 and E_max values of 153 μM and 135 ± 26%, respectively, whereas the corresponding values in 2087101-treated oocytes were 6 μM and 166 ± 13%, respectively.

The effect of 2087101 on the pharmacological profile of a number of partial nAChR agonists was also compared in

Fig. 3. Potentiation of human α4β2, α4β4, and α7 nAChRs but not α3β2 or α3β4 nAChRs expressed in Xenopus oocytes by 2087101, 1078733, and 2087133. A, example traces showing inward currents in voltage-clamped Xenopus oocytes expressing nAChRs in response to superfusion with a submaximal (EC10–30) concentration of acetylcholine in the absence or presence of 3 μM 2087101, 1078733, or 2087133, as indicated. B, concentration-dependent potentiation of submaximal (EC10–30) acetylcholine-induced responses in Xenopus oocytes expressing human α4β2, α4β4, and α7 nAChRs by 2087101 (30 nM to 30 μM), as indicated. Data are mean ± S.E.M. from at least three similar experiments. For heteromeric nAChRs, potentiators were coapplied with agonist, whereas for α7 nAChRs, oocytes were preincubated with potentiator for 2 min before application of acetylcholine.

Fig. 4. Nicotinic receptor agonists display increased potency and maximal efficacy in the presence of 2087101. A, epibatidine concentration-response curves in mammalian cells stably expressing human α3β4, α4β2, α4β4, or α7 nAChRs measured in the absence (○) or presence of 1 (□) or 3 (▲) μM 2087101, as indicated. Cells were preincubated with 2087101 for ~10 min before agonist application. B, acetylcholine concentration-response curves in Xenopus oocytes expressing human α4β2 nAChRs measured in the absence (○) or presence (■) of 1 μM 2087101. C, concentration-response curves for nicotine, TC-2559, and cytisine in HEK cells stably expressing human α4β2 nAChRs measured in the absence (○) or presence (■) of 1 μM 2087101, as indicated. Cells were preincubated with 2087101 for ~10 min before agonist application. For all experiments, data are mean ± S.E.M. from at least three similar experiments.
The potency and efficacy of epibatidine at α4β2, α4β4, and α7 nAChRs stably expressed in mammalian cells are significantly increased by 2087101.

Fluo-3-AM-loaded cells stably expressing human α3β4, α4β2, α4β4, and α7 nAChRs were challenged with epibatidine in the presence or absence of 1 μM 2087101 (α4β2, α4β4) or 3 μM 2087101 (α3β4, α7) as indicated. Cell population Ca2+ measurements were recorded using a FLIPR. Each value represents the mean ± S.E.M. from at least four independent experiments.

<table>
<thead>
<tr>
<th>nAChr Subtype</th>
<th>Control</th>
<th>Treated with 2087101</th>
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<tr>
<td></td>
<td>EC50 (nM)</td>
<td>Emax (%)</td>
</tr>
<tr>
<td>α3β4</td>
<td>11 ± 1</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>α4β2</td>
<td>15.6 ± 1.5</td>
<td>103 ± 7</td>
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<tr>
<td>α4β4</td>
<td>3.8 ± 0.9</td>
<td>99 ± 6</td>
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<tr>
<td>α7</td>
<td>79 ± 14</td>
<td>133 ± 9</td>
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<tr>
<td>** p &lt; 0.01.</td>
<td>*** p &lt; 0.05.</td>
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HEK-293 cells stably expressing α4β2 nAChRs. The effects of 1 μM 2087101 on TC-2559, cytisine, and nicotine-induced concentration response curves was assessed after ~10-min preincubation with the potentiator and compared with the profile in its absence (Fig. 4C). Both potency and efficacy of these partial agonists were significantly increased in the presence of 2087101 (Table 2).

**Comparison of Potentiation of α4β2 nAChRs by 2087101 and Galantamine.** The activity of 2087101 was also compared with that of a known nicotinic potentiator, galantamine (Samochcki et al., 2003). The effect of the two potentiators on epibatidine-induced responses was assessed in HEK-293 cells stably expressing α4β2 nAChRs after an ~10-min preincubation with a range of agonist concentrations (30 nM to 10 μM). In the presence of 1 μM galantamine, the potency (EC50 = 17 ± 2 versus 28 ± 2 nM) but not the efficacy (Emax = 93 ± 1 versus 89 ± 2%) of epibatidine were significantly increased (Fig. 5A). The concentration window for potentiation by galantamine of the submaximal (EC50) epibatidine-induced responses was relatively narrow, as previously reported (Samochcki et al., 2003), with significant potentiation only seen at 1 μM galantamine (Fig. 5B). In contrast, significant potentiation of submaximal (EC50) epibatidine-induced responses by 2087101 was observed over a wider concentration range (Fig. 5B).

**Intrinsic Agonist Properties of 2087101 on nAChRs.** At concentrations of 10 to 100 μM, compounds 2087101, 1078733, and 2087133 displayed intrinsic agonist properties at α4β2, α4β4, and α2β4 nAChRs expressed in HEK-293 cells. Agonism was most apparent for α4β4 nAChRs: 2087101-evoked responses were 55 ± 2 and 42 ± 6% of a maximally effective concentration of epibatidine at 30 and 100 μM 2087101, respectively. Smaller agonist effects of 2087101 were also observed on α4β2 nAChRs (2 ± 1 and 4 ± 2% of epibatidine at 30 and 100 μM, respectively) and on α2β4 nAChRs (13 ± 1 and 15 ± 3% of epibatidine at 30 and 100 μM, respectively). In the FLIPR experiments, the agonistic activity of 2087101 (100 μM) was completely prevented by the noncompetitive antagonist mecamylamine (100 μM), confirming that they were mediated by nAChR activation rather than nonspecific mechanisms (data not shown).

**Activation of nAChRs by the potentiators was further confirmed by electrophysiological recordings in oocytes.** Figure 6 shows the agonistic properties of 30 μM 1078733, compared with a maximal concentration of ACh. Furthermore, we confirmed that the agonist responses are mediated by the expressed nAChRs since both the competitive antagonist dHβE (10 μM, Fig. 6) and the noncompetitive antagonist mecamylamine (data not shown) completely and reversibly blocked the potentiator’s effects.

**Assessing the Mechanism of Action of 2087101,** 1078733, and 2087133-Induced Potentiation of nAChRs. More experiments were designed to further establish whether the potentiation of nAChRs by 2087101, 10787133, and 2087133 was through a direct competitive or allosteric action on nAChRs or not via an indirect mechanism.

The ability of the three compounds to displace [3H]epibatidine from α4β2, α4β4, and α3β4 nAChRs was established and compared with displacement by a standard agonist, cytisine. At concentrations up to 10 μM, 2087101, 10787133, and 2087133 did not displace [3H]epibatidine from α4β2, α4β4, or α3β4 nAChRs (data not shown), whereas cytisine generated the expected Kd values for each of the receptor subtypes tested (2.1, 2.63, and 551 nM for α4β2, α4β4, or α3β4 nAChRs, respectively).

Since some nAChR potentiators are reported to act via second messenger systems, the effects of a panel of common second messenger and G protein disruptors was studied. Acute pretreatment (10~20 min) of HEK-293 cells stably expressing α4β2 nAChRs with 1 μM PMA, 50 μM H7, 100 nM staurosporine, or 10 μM forskolin to modulate protein kinases and 100 nM calyculin A or 100 nM okadaic acid to modulate protein phosphatases, respectively, failed to prevent potentiation of epibatidine-induced responses by 1 μM 2087101, as shown by a comparable decrease in EC50 values and increase in Emax values in control versus treated cells (Fig. 7). Additionally, chronic pretreatment (16~20 h) of HEK-293 cells stably expressing α4β2 nAChRs with 200 ng/ml pertussis toxin or 500 ng/ml cholera toxin to disrupt G protein signaling, respectively, failed to prevent potentiation of epibatidine-induced responses by 1 μM 2087101 (Fig. 7).

**2087101 Does Not Potentiate Chimeric α7/5-HT3 Receptors.** To determine whether the extracellular N-terminal domain of the α7 nAChR was required for potentiation by 2087101, potentiation of agonist-induced responses was assessed using a chimeric α7/5-HT3 receptor composed of the N terminus of the human α7 nAChR and the remainder of the mouse 5-HT3 receptor (Eisele et al., 1993; Craig et al., 2004). Potentiation of epibatidine-induced responses by 2087101 at α4β2, α4β4, and α2β4 nAChRs expressed in HEK-293 cells.
chimeric a7/5-HT3 receptors stably expressed in HEK-293 cells was assessed after an ~10-min preincubation with potentiatior alone. 2087101, at concentrations up to 10 μM, failed to modulate agonist-induced responses at chimeric a7/5-HT3 receptors (Fig. 8). Interestingly, also the intrinsic agonist effects at high potentiatior concentrations were absent in the chimera. The absence of effects on the chimera is not due to major structural and functional deficits of the chimera itself since we have shown before that this chimera can be activated and potentiated by nicotinoy agonists and nicotinoy potentiators like 5-hydroxy-indol, respectively (Craig et al., 2004).

**Selectivity Profile of Compounds 2087101, 1078733, and 2087133.** To verify that compounds 2087101, 1078733, and 2087133 were selective potentiators of nAChRs, the compounds were profiled across a panel of ligand- and voltage-gated ion channels (Fig. 9). Compound activity was assessed on human recombinant 5-HT3 receptors stably expressed in HEK-293 cells and at rat native AMPA receptors, NMDA receptors, GABA_A receptors, and voltage-gated Ca^{2+} and Na^+ channels endogenously expressed in cortical neurons. Activity was compared with the effects seen on human recombinant a4β2 nAChRs stably expressed in HEK-293 cells. Addition of 3 μM 2087101, 1078733, or 2087133 failed to evoke an agonist response at any of the ion channels tested for cross-reactivity (data not shown). In addition, no significant potentiating effect was observed on responses to 1 μM 5-HT (5-HT3 receptors), 3 μM AMPA (AMPA receptors), 10 μM NMDA (NMDA receptors), 25 mM K^+ (voltage-gated Ca^{2+} channels), and 3 μM veratridine (voltage-gated Na^+ channels) after an ~10 min preincubation with 3 μM 2087101, 1078733, or 2087133 (Fig. 9).

**Acetylcholine Esterase Inhibition.** The activity of compound 2087101 as an ACh esterase inhibitor was also tested and compared with a known ACh esterase inhibitor, neostigmine. At 3 μM, 2087101 caused only minor (21%) inhibition of human ACh esterase stably expressed in HEK-293 cells (data not shown), whereas neostigmine generated an IC_{50} of 38 nM.

**Discussion**

We report the discovery of a novel class of nicotinic acetylcholine receptor potentiators, exemplified by compounds 2087101, 1078733, and 1078733. These compounds selectively potentiate human recombinant α7, α2β4, α4β2, and α4β4, but not α3- or α1-containing nAChRs or other ion channels.

The profile of nAChR potentiation, as exemplified by compound 2087101, is unique, even if it shares some features with other allosteric nAChR potentiators. For example, 2087101 increases both the potency and the efficacy of nAChR agonists at α4β2, α4β4, and α7 nAChRs. Potentiation by 2087101 is independent of agonist type because potentiation of a variety of full and partial agonists is observed, and it is present, albeit to a lower degree, at supramaximal agonist concentrations. As previously reported (Samochcki et al., 2003) and replicated in this study, galantamine, a known allosteric modulator of nAChRs, increases the apparent affinity of α4β2 nAChRs for agonists. However, unlike 2087101, galantamine only potentiates submaximal agonist-induced responses not having significant effects on agonist efficacy. This is a profile shared by 17β-estradiol, another allosteric nAChR modulator, which is also reported to potentiate submaximal agonist responses at α4-containing nAChRs, thereby increasing agonist potency without affecting agonist efficacy (Paradiso et al., 2001; Curtis et al., 2002). In contrast, ethanol, a direct nAChR allosteric modulator, and nefiracetam, an indirect nAChR potentiator, potentiate α4β2 nAChRs even at saturating concentrations of agonist, leading to an increase in agonist efficacy, whereas the EC_{50} for agonist remains relatively unaffected.

The mechanism of nAChR potentiation by compounds 2087101, 10787133, and 1078733 is likely to be a direct allosteric interaction on the nAChR. Firstly, potentiation is non-competitive because it is independent of agonist concentration, and at concentrations required for nAChR potentiation,
potentiation of systems does not prevent nAChR potentiation. In contrast, pharmacological modulation of common second messenger systems prevents potentiation (Oyaizu and Narahashi, 1999). Thirdly, at concentrations higher than those required for potentiation, 2087101, 2087133, and 1078733 exhibit intrinsic agonist activity. This is consistent with the “allosteric model” of ligand-gated channels (Hogg et al., 2005) and is indeed a feature shared with other allosteric ion channel modulators, including 17β-estradiol, which evokes single-channel nAChR activity (Pereira et al., 1994). In addition, the anthelmintic agent levamisole, which potentiates channel nAChR activity (Pereira et al., 1994). In addition, the compounds do not displace 3H agonist binding. Secondly, pharmacological modulation of common second messenger systems does not prevent nAChR potentiation. In contrast, potentiation of α4β2, muscle and α3-containing nAChRs by nefracetam requires G protein kinase C and G/protein kinase A, respectively, because disruption of these second messenger pathways prevents potentiation (Oyaizu and Narahashi, 1999). Thirdly, at concentrations higher than those required for potentiation, 2087101, 2087133, and 1078733 exhibit intrinsic agonist activity. This is consistent with the “allosteric model” of ligand-gated channels (Hogg et al., 2005) and is indeed a feature shared with other allosteric ion channel modulators, including 17β-estradiol, which evokes single-channel nAChR activity (Pereira et al., 1994). In addition, the anthelmintic agent levamisole, which potentiates α3β2 and α3β4 nAChRs in the micromolar concentration range, is also reported to display some weak partial agonist activity at millimolar concentrations (Levandoski et al., 2003).

The allosteric binding site for galantamine has been mapped to the N-terminal region of nAChRs by use of monoclonal antibodies and a chimeric α7/5-HT₃ receptor (Maelicke et al., 2000; Samochocki et al., 2003). Use of chimeric α4 and α3 nAChRs also allowed the site of potentiation for 17β-estradiol to be mapped to the C-terminal domain of the α4 nAChR (Curtis et al., 2002). In the present study, we also used a chimeric α7/5-HT₃ receptor to determine whether the allosteric site for potentiation by 2087101 resides in the extracellular N-terminal domain of the nAChR. The chimera is composed of the N-terminal extracellular domain of the α7 nAChR and the transmembrane and C-terminal domains of the 5-HT₃ receptor and is reported to display a pharmacological profile similar to wild-type α7 nAChRs (Eisele et al., 1993; Craig et al., 2004). This chimera has previously been shown to be potentiated by both 5-hydroxy-indole (Craig et al., 2004) and galantamine (Samochocki et al., 2003). However, at variance with findings with these modulators, 2087101 did not potentiate agonist-induced responses at chimeric α7/5-HT₃ receptors, suggesting that its binding site might reside in regions downstream of the N terminus or, alternatively, that these regions are needed to achieve the selective conformational changes induced by 2087101.

At high concentrations, 2087101, 2087133, and 1078733...
caused inhibition, even on nAChRs where no potentiation was evident. This is in line with previous reports of additional inhibitory actions seen with steroids (Paradiso et al., 2001), galantamine (Samochcki et al., 2003), and levamisole (Levandoski et al., 2003). The inhibitory action of these potentiators is thought to be largely mediated through sites distinct from those mediating potentiation. For galantamine and levamisole, this is proposed to be through a nonselective block of the pore, whereas for steroids, the site-mediating inhibitory action is unclear but has been shown to lie outside of the C-terminal region mediating 17β-estradiol potentiation (Paradiso et al., 2001).

The profile of 2087101, 2087133, and 1078733 as selective potentiators of central, but not peripheral, ganglionic α3- or muscle α1-containing nAChRs or of other ion channels such as NMDA, AMPA, GABA_A, and voltage-gated sodium or calcium channels compares very favorably with that of currently known nAChR potentiators. For example, galantamine is reported to nonselectively potentiate central α4β2, α7, α6β4, and peripheral α3β4 nAChRs (Samochcki et al., 2003), as well as NMDA receptors (Moriguchi et al., 2004). Because galantamine also possesses anti-AChE activity, the interpretation of its mechanism of action, although not of its clinical efficacy, is further complicated. Nefiracetam is reported to potentiate α4β2-, α7-, and α3-containing and muscle nAChRs (Oyaizu and Narahashi, 1999) as well as GABA_A receptors (Huang et al., 1996) and voltage-gated calcium channels (Yoshii and Watabe, 1994). Likewise, antihistamines such as levamisole and pyrental potentiate peripheral α3-containing and muscle nAChRs (Levandoski et al., 2003), and ethanol nonselectively acts on nAChRs among other ion channels including NMDA receptors, GABA_A receptors, and voltage-gated calcium channels (Zuo et al., 2001). Although 17β-estradiol displays selectivity for α4 over α3-containing nAChRs (Curtis et al., 2002), it is also reported to potentiate kainate receptors (Gu and Moss, 1996). In addition, 17β-estradiol obviously interacts with estrogen receptors.

The profile of these newly described compounds as selective potentiators of the two main central nAChR subtypes, α7 and α4β2 nAChRs, is intriguing. Activation of either α7 or α4β2 nAChRs with subtype-selective agonists produces pro-cognitive effects, demonstrating a role for both subtypes in modulation of cognitive function (Lippiello et al., 1996; Levin and Rezvani, 2000). Selective action of these potentiators at central, but not peripheral ganglionic α3- or muscle α1-containing nAChRs, may give the specificity required to avoid undesirable side effects. Hurst et al. (2005) and Sher et al. (2005) recently described different and new selective α7 nAChR potentiators that significantly increase potency and efficacy of agonists at recombinant and native α7 receptors. On the other hand, the lack of selectivity between α7 or α4β2 nAChRs could be advantageous for different disease pathologies in which both α4β2 and α7 nAChRs have been implicated (Leonard et al., 2000; Court et al., 2001; Quik and Kulak, 2002). In any event, these compounds should prove useful as tools to establish whether nAChR potentiators can produce comparable pro-cognitive enhancements as direct nAChR agonists or AChE inhibitors. In addition, the compounds should help clarify whether nAChR potentiators display additional advantages, such as the induction of less desensitization, compensation, or tolerance (because potentiation occurs only when endogenous agonist is present) or if the compounds display a favorable safety profile, as previously suggested (Maelicke et al., 2000), and ultimately if this type of compound has a therapeutic application.

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References


Cheng YC and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_50) of an enzymatic reaction. Eur J Pharmacol 29:273–286.


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