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3'-Fluoro substitution in the pyridine ring of epibatidine improves selectivity and efficacy for $\alpha 4\beta 2$ versus $\alpha 3\beta 4$ nAChRs

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Abstract

The analog of epibatidine having a fluoro- substituent at the 3' position of the pyridine ring has been recently developed and shown to possess binding affinity in the pM range to $\alpha 4\beta 2$ nAChRs and in the nM range to $\alpha 7$ nAChRs and to exhibit potent agonist activity in nicotine-induced analgesia tests. Here we used patch-clamp technique in a whole-cell configuration to compare functional activity of 3'-fluoroepibatidine to that of epibatidine by itself on recombinant $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ neuronal nAChRs. The agonist effect of ($\pm$)-epibatidine was partial and yielded comparable EC₅₀s of 0.012 μ M (72% efficacy) and 0.027 μ M (81% efficacy) at $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs, respectively, but was full at $\alpha 7$ nAChRs with an EC₅₀ of 4.8 μ M. Testing of the analog at different concentrations revealed that it acts as a full agonist with an EC₅₀ of 0.36 μ M at $\alpha 4\beta 2$ nAChRs and induces partial agonist effect (66% efficacy) at $\alpha 7$ nAChRs with an EC₅₀ of 9.8 μ M and an IC₅₀ corresponding to 225 μ M. In contrast, the analog caused only 24% maximal activation at the range of concentrations from 0.1–100 μ M and, in addition, induced an inhibition of $\alpha 3\beta 4$ nAChR function with an IC₅₀ of 8.3 μ M. Our functional data, which are in agreement with previous binding and behavioral findings, demonstrate that 3'-fluoro substitution in the pyridine ring of epibatidine results in an improved pharmacological profile as observed by an increased efficacy and selectivity for $\alpha 4\beta 2$ versus $\alpha 3\beta 4$ nAChRs.

Keywords

recombinant neuronal nicotinic receptors; epibatidine; epibatidine analogs; whole-cell; nicotine-induced analgesia; binding

Introduction

The natural alkaloid epibatidine, while showing high affinity to neuronal nAChRs and an analgesic effect in animal models of pain, produces many negative side effects including toxicity, hypertension, respiratory paralysis and seizures due, in part, to its low selectivity for

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central nAChRs (Badio and Daly, 1994; Daly et al., 2000; Sullivan and Bannon, 1996; Sullivan et al., 1995). Neuronal nAChRs consist of various combinations of $\alpha 2$ - $\alpha 10$ and $\beta 2$ - $\beta 4$ subunits and are differently distributed in various regions of the mammalian nervous system with respect to their subunit composition (Dani and Bertrand, 2007; Gotti et al., 2006b; Perry et al., 2002). The $\alpha 4\beta 2$ is a major nAChR subtype of the central nervous system (Flores et al., 1992; Whiting and Lindstrom, 1987), while $\alpha 3\beta 4$, though detected in some brain regions, predominates in the periphery (Flores et al., 1996; Quick et al., 1999), and $\alpha 7$ nAChRs are present in both central and peripheral regions of the nervous system (Dickinson et al., 2008; Genzen et al., 2001; Keath et al., 2007; Lips et al., 2006; Woollorton et al., 2003). The spatial distribution reflects an involvement of $\alpha 4\beta 2$ and $\alpha 7$ nAChR subtypes in crucial physiological functions such as e.g. learning, memory, pain control and neuroprotection (Damaj et al., 2000; Levin et al., 2002; Liu et al., 2001; Marubio et al., 1999; Picciotto and Zoli, 2008; Picciotto et al., 1995; Wu et al., 2004) versus ganglionic transmission and control of autonomic functions for $\alpha 3\beta 4$ nAChRs (De Biasi, 2002; Flores et al., 1996; Wang et al., 2003). Therefore, a goal of many laboratories is to develop epibatidine analogs with improved safety profile, selectivity and efficacy towards central nAChRs that can be potentially utilized for the treatment of a number of pathological states of the nervous system associated with dysfunction of nAChRs (Carroll et al., 2002; Huang et al., 2005; Wei et al., 2005). Structural modifications of the epibatidine molecule lead to variable alterations in its pharmacological effects on nAChRs (Carroll, 2004; Romanelli et al., 2007; Spang et al., 2000), e.g. replacement of the 2'-chloro present in epibatidine by fluorine and addition of phenyl group with 4-nitro substitution provides a potent competitive nAChR antagonist with high selectivity for the $\alpha 4\beta 2$ nAChR subtype (Abdrakhmanova et al., 2006). The epibatidine analog with a fluoro- (electron-withdrawing) substituent at the 3' position of the pyridine ring and epibatidine by itself (Fig. 1) have been previously characterized in binding assays in rat brain and evaluated in acute nicotine-induced analgesia tests (Carroll et al., 2001; Carroll et al., 2002). The 3'-fluoro substituted analog possessed high binding affinity to native $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, and exhibited potent antinociceptive activity in both hot-plate and tail-flick pain tests with the K_i and ED_{50} parameters similar to those determined for epibatidine by itself. Our patch-clamp experiments were aimed to investigate whether the fluoro- substitution at the 3' position of the pyridine ring of epibatidine induces desirable changes in the efficacy and subtype selectivity for central neuronal nAChRs. The preliminary report on this work has already appeared as (Abdrakhmanova et al., 2007).

Methods

Cell transfection and culture

Stably transfected HEK 293 cells expressing rat $\alpha 3\beta 4$ or rat $\alpha 7$ and SH-EP1 cells expressing human $\alpha 4\beta 2$ neuronal nAChRs, respectively, were prepared as described previously (Eaton et al., 2003; Moaddel et al., 2008; Xiao et al., 1998). All three cell lines were maintained at 37°C with 5% CO₂ in the incubator. Growth medium for HEK 293 cells was minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The stably transfected cell line was raised in selective growth medium containing 0.7 mg/ml of Geneticin (Invitrogen Corp, Carlsbad, CA). Growth medium for SH-EP1 cells was Dulbecco's Modified Eagle's medium with high glucose supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 8 mM L-glutamine, 1 mM sodium pyruvate, and 0.25 µg/ml amphotericin (all from Invitrogen Corp, Carlsbad, CA). This stably transfected cell line was raised in selective medium containing 0.5 mg/ml zeocin (Invitrogen) and 0.4 mg/ml hygromycin B (Roche Diagnostics Corp, Indianapolis, IN). RT-PCR analysis was used to confirm expression of nAChR subunit messages in the cells and immunoprecipitation-Western analyses using solubilized membrane samples from transfected cells clearly indicated that subunits were expressed as a protein and

assembled together. Control experiments excluded possible activation of muscarinic ACh receptors by ACh application in both cell lines. Difference in species of the nAChRs used in this study (rat versus human) is due to current unavailability to us of these nAChR subtypes of the same specie that would be functional and expressed in the cells at sufficiently high level. Rat and human nAChR subunits share 82–95% sequence identity, and when present in neuronal nAChR receptors of the same subunit composition provide them with numerous similarities in their properties (Albuquerque et al., 2000).

Whole-cell current recording

Functional expression of nAChRs was evaluated in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Molecular devices, Sunnyvale, CA). The patch electrodes, pulled from borosilicate glass capillaries (Sutter Instrument Company, Novato, CA), had a resistance of 2.5–3.5 MΩ when filled with internal solution containing 110 mM Tris phosphate dibasic, 28 mM Tris base, 11 mM EGTA, 2 mM MgCl₂, 0.1 mM CaCl₂ and 4 mM Mg-ATP (pH adjusted to 7.3 with Tris base) (Wu et al., 2004). In some cells ~85% of electrode resistance was compensated electronically, so that the effective series resistance in the whole-cell configuration was accepted when less than 20 MΩ. Stably transfected HEK and SH-EP1 cells were studied for 2 to 3 days after plating the cells on the 15-mm round plastic cover slips (Thermanox, Nalge Nunc, Naperville, IL). Generation of voltage-clamp protocols and acquisition of the data were carried out using pCLAMP 9.0 software (Molecular Devices). Sampling frequency was 5 kHz and current signals were filtered at 5 or 10 kHz before digitization and storage. All experiments were performed at room temperature (22–25°C).

Application of drugs and Perfusion system

Cells plated on cover slips were transferred to an experimental chamber mounted on the stage of an inverted microscope (Olympus IX50, Olympus Corporation, Tokyo, Japan) and were bathed in a solution containing 140 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 25 mM D-glucose, 10 mM HEPES and 2 mM CaCl₂ (pH adjusted to 7.4 with Tris base). The experimental chamber was constantly perfused with control bathing solution (1–2 ml/min). The amplitude and time course of currents mediated by neuronal nAChRs is highly dependent on the speed of drug application. The high speed solution exchange system, HSSE-2 (ALA Scientific Instruments, Westbury, NY), is able to switch rapidly between control and four test solutions delivered through two output tubes which face each other at 90° in the same plane. Under optimal conditions, the delay in switching between solutions is ~10 ms. Data presented herein were obtained through subtraction from the leak current.

Data analysis

The peak amplitude and the exponential decay time constant (τ) of the whole-cell currents were determined using the pCLAMP 9.0 program. EC₅₀, IC₅₀, and the nH values were determined with the Origin 5.0 program (Microcal, North Hampton, MA). To determine the EC₅₀ values, the epibatidine or 3'-fluoro analog-induced responses were recorded at –80 mV and normalized to the amplitude of the current elicited by ACh alone at its saturating concentration (1 mM) in each cell. Values were plotted against the concentration of the tested agonist on a logarithm scale and fitted with an equation: $I = I_{\max} / (1 + (EC_{50} / [\text{agonist}])^{nH})$, where I is the current amplitude at the agonist concentration [agonist], $I_{\max}$ is the maximum current, EC₅₀ is the concentration of the agonist eliciting a half maximum response and nH is the Hill coefficient. Peak amplitude of the currents induced by epibatidine or its 3'-fluoro analog was normalized to the maximum ACh (1mM) whole-cell response in each examined cell. IC₅₀ values corresponded to the concentration of inhibiting agent causing a 50% reduction in the current evoked by a pulse of ACh at the concentration near the EC₅₀ value for each tested nAChR subtype. The ACh-evoked currents in the presence of the 3'-fluoro analog were measured at

–80 mV and normalized to the amplitude of the current elicited by ACh alone. Values were plotted against the concentrations of the inhibitor on a logarithm scale and fitted with an equation: $I = I_{\max}/(1 + (IC_{50}/[\text{antagonist}])^{nH})$, where I is the current amplitude at the antagonist concentration $[\text{antagonist}]$, $I_{\max}$ is the maximum current, and nH is the Hill coefficient. Results are presented as the mean $\pm$ S.E.M for the number of cells (n) or as averaged means.

Drugs

ACh chloride, ($\pm$)-epibatidine, dihydro- β -erythroidine (DH β E), methyllycaconitine (MLA), mecamlamine and salts were purchased from Sigma Aldrich (Atlanta, GA). The 3'-fluoro-epibatidine (for structure see Fig. 1) was racemic and synthesized as previously reported (Carroll et al., 2002).

Results

In the first set of experiments we tested the agonist effect of a selective nAChR ligand epibatidine (Badio and Daly, 1994) on three major subtypes of neuronal nAChRs: $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$. Peak amplitude of the whole-cell currents elicited by the pulse application of ($\pm$)-epibatidine of 200 ms duration for both $\alpha 4\beta 2$ and $\alpha 3\beta 4$ and 100 ms duration for $\alpha 7$ was normalized to that induced by the full agonist ACh at saturating 1 mM concentration in each examined cell (Fig. 2A). The data analysis indicated that ($\pm$)-epibatidine behaved as a potent partial agonist at $\alpha 4\beta 2$ nAChRs with an EC_{50} value of $0.012 \pm 0.001 \mu\text{M}$ and an intrinsic efficacy of $72.0 \pm 3.0\%$ (relative to maximum response produced by 1 mM ACh) ($nH=1.1 \pm 0.2$; $n=3-4$) (Fig. 2B). ($\pm$)-Epibatidine acted in a similar manner at $\alpha 3\beta 4$ nAChRs with a comparable efficacy of $81.2 \pm 7.2\%$ ($nH=2.7 \pm 1.3$; $n=3-5$) although with slightly lower potency (EC_{50} of $0.027 \pm 0.003 \mu\text{M}$). In contrast, ($\pm$)-epibatidine appeared to act as a full ($100.0 \pm 4.8\%$) agonist on $\alpha 7$ nAChRs with a higher EC_{50} value of $4.8 \pm 0.8 \mu\text{M}$ ($nH=0.9 \pm 0.1$; $n=3-4$) than in $\alpha 4\beta 2$ or $\alpha 3\beta 4$ nAChRs (Fig. 2B).

Next, we tested, under similar experimental conditions, the agonist effect of the 3'-fluoroepibatidine on $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChR subtypes. Peak amplitude of the whole-cells currents elicited by the 3'-fluoro analog was normalized to that induced by 1 mM ACh in each examined cell. Application of the 3'-fluoro analog at different concentrations in three nAChR subtypes revealed that the compound behaved as a full agonist with an intrinsic efficacy of $96.6 \pm 6.9\%$ (relative to maximum response produced by 1 mM ACh) and an EC_{50} value of $0.36 \pm 0.09 \mu\text{M}$ at $\alpha 4\beta 2$ nAChRs ($nH=0.6 \pm 0.1$; $n=3-4$). In contrast, the analog induced only $23.5 \pm 3.2\%$ activation as maximum in $\alpha 3\beta 4$ nAChRs when tested at 0.1–100 μM concentration range ($n=3-7$). The analog-evoked whole-cell responses at $\alpha 7$ nAChRs appeared to be also concentration-dependent and were activated with an EC_{50} value of $9.8 \pm 1.5 \mu\text{M}$ ($nH=1.0 \pm 0.1$) and maximal efficacy of $65.9 \pm 11.9\%$ (relative to maximum response produced by 1 mM ACh) at concentrations above 60 μM ($n=3-6$) (Fig. 3A). The superimposed current traces in Figure 3B illustrate the agonist effect of 10 μM 3'-fluoro substituted analog compared to that of 1 mM ACh in three representative cells expressing $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChR subtypes. While full current activation was achieved in $\alpha 4\beta 2$ nAChRs ($96.6 \pm 6.9\%$), 2.5-fold less activation was observed in $\alpha 7$ nAChRs ($37.8 \pm 3.3\%$), and only a small response was induced at $\alpha 3\beta 4$ nAChRs ($16.4 \pm 4.4\%$) ($n=4-6$). It is worth noting that in cells expressing $\alpha 4\beta 2$ nAChRs, the whole-cell currents induced by the 3'-fluoro analog at 10 μM concentration, while being of about equal peak amplitude to those activated by 1 mM ACh in the same cell, were characterized by slower decay kinetics (Fig. 3B, top panel) so that the time constant of the decay when fit with single exponential function (τ) was longer by $32.7 \pm 6.1\%$ in the presence of the 3'-fluoro analog than in the presence of ACh ($n=3$).

Currents induced by the 3'-fluoro substituted analog in two cell lines expressing $\alpha 4\beta 2$ or $\alpha 7$ nAChRs were abolished by the $\alpha 4\beta 2$ nAChR competitive antagonist DH β E (5 μM) and the

$\alpha 7$ nAChR competitive antagonist MLA (10 nM), respectively (n=3) (Fig. 3C). The analog-induced current was blocked by mecamylamine (20 μ M) in $\alpha 3\beta 4$ nAChRs (n=3). No current was induced by the analog by itself in non-transfected SH-EP1 or HEK 293 cells (n=3). These control experiments confirm that the currents activated by 3'-fluoroepibatidine are mediated by neuronal nAChRs.

Based upon the above data, the inhibitory potency of the 3'-fluoro substituted analog was also assessed for $\alpha 7$ and $\alpha 3\beta 4$ nAChRs. The cell under recording was exposed to the EC₅₀ concentration of ACh, determined previously for each nAChR subtype and corresponding to 280 μ M for $\alpha 7$ (Abdrakhmanova et al., 2007) and 100 μ M for $\alpha 3\beta 4$ nAChRs (Zhang et al., 1999), and later with a 30 s intervals to ACh at the same concentration in the presence of various concentrations of the analog. The peak amplitude of ACh-induced currents was decreased more potently in $\alpha 3\beta 4$ than in $\alpha 7$ nAChRs with the IC₅₀ values of $8.3 \pm 0.6 \mu$ M ($nH=1.7 \pm 0.2$; n=3–5) and $224.7 \pm 38.7 \mu$ M ($nH=1.3 \pm 0.2$; n=3–4), respectively (Fig. 4A). Figure 4B illustrates typical ACh(EC₅₀)-induced currents recorded from $\alpha 7$ - and $\alpha 3\beta 4$ -expressing cells voltage-clamped at the holding potential of –80 mV in the absence and presence of 3'-fluoro analog at the concentrations close to determined IC₅₀ value for each nAChR subtype.

A rebound current, currently considered to be due to an agonist-induced open-channel block in nAChRs (Drapeau and Legendre, 2001), was consistently observed in $\alpha 3\beta 4$ -expressing cells at the negative holding potentials upon rapid withdrawal of ACh applied at high 1mM concentration (Fig. 2A&3B). This observation is in agreement with our earlier studies carried out on the same cell line (Abdrakhmanova et al., 2002; Zhang et al., 1999). Interestingly, the rebound current also occurred upon a rapid withdrawal of the 3'-fluoro analog co-applied with 100 μ M ACh at the concentrations of the analog $\geq 1 \mu$ M (Fig. 4B). Similar to the data reported by Liu et al. (2008), a less pronounced rebound current was also noticeable in $\alpha 4\beta 2$ -expressing cells upon rapid withdrawal of ACh or 3'-fluoroepibatidine applied at high saturating concentrations (Fig. 3B).

Discussion

Testing of the agonist effect of ($\pm$)-epibatidine by itself on three major neuronal nAChR subtypes revealed that it acts as a potent partial agonist on human $\alpha 4\beta 2$ and rat $\alpha 3\beta 4$ nAChRs, expressed in SH-EP1 and HEK 293 cells, respectively, with comparable efficacy and EC₅₀s corresponding to 12 and 27 nM, respectively, but appears to be a full and less potent agonist (EC₅₀ was 4.8 μ M) for rat $\alpha 7$ nAChRs expressed in HEK 293 cells (see Table 1, Fig. 2). The higher potency of epibatidine in recombinant human $\alpha 4\beta 2$ than rat $\alpha 7$ nAChRs determined in our functional experiments is in agreement with its earlier reported higher affinity to native $\alpha 4\beta 2$ than to $\alpha 7$ nAChRs in the binding assays performed on the rat brain (Carroll et al., 2002). Avalos et al. (2002) estimated EC₅₀s of 14 and 128 nM for (+)-epibatidine in rat $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChRs, respectively, expressed in *Xenopus* oocytes. Buisson et al. (2000) determined, using patch-clamp technique, a high affinity component with an EC₅₀ of 3.4 nM for ($\pm$)-epibatidine in rat $\alpha 4\beta 2$ nAChRs expressed in HEK tsA 201 cells, and an EC₅₀ of 1.9 μ M was estimated by Criado et al. (2005) for bovine $\alpha 7$ nAChRs expressed in *Xenopus* oocytes. All together, these functional data confirm that epibatidine is a potent neuronal nAChR agonist with a rank order of potency for tested nAChR subtypes, $\alpha 4\beta 2 \geq \alpha 3\beta 4 > \alpha 7$, thus, specifically suffering from a lack of selectivity for $\alpha 4\beta 2$ versus $\alpha 3\beta 4$ nAChR subtype. In both our experiments and the above-mentioned studies, epibatidine behaved as a partial agonist in $\alpha 4\beta 2$ nAChRs but as a full agonist in $\alpha 7$ nAChRs. The 81% efficacy of epibatidine in rat $\alpha 3\beta 4$ nAChRs, that was determined in our study, appears to be closer to 100% efficacy reported by Lindovsky et al. (2008) and Meyer et al. (2001), using patch-clamp and $^{86}\text{Rb}^+$ efflux techniques, respectively, for this nAChR subtype but differs from 39% efficacy found by

Avalos et al. (2002). The variability in the data, though all obtained at the room temperature, could be due to different expression systems, methods and protocols used in these studies.

Human $\alpha 4\beta 2$ nAChRs used in this study (Eaton et al., 2003) are activated by ACh and epibatidine with the EC_{50} values corresponding to 20 μM (Abdrakhmanova et al., 2006) and 0.012 μM and the efficacies close to 100% and 72%, respectively. These pharmacological parameters are rather similar to those reported for high-affinity human $\alpha 4\beta 2$ nAChRs with $(\alpha 4)_2(\beta 2)_3$ stoichiometry, in which, when expressed in *Xenopus* oocytes, ACh activated currents with an EC_{50} of 4 μM and 100% efficacy and epibatidine showed the EC_{50} close to 0.020 μM and 72% efficacy (Moroni et al., 2006).

Examination of functional activity of the 3'-fluoroepibatidine in the same three nAChR subtypes revealed that the addition of a 3'-fluoro substituent to epibatidine induced a number of major changes in its pharmacological profile (Fig. 3 & 4; Table 1). Specifically, the 3'-fluoro substitution in epibatidine resulted in an analog that behaved not as a partial but as a full agonist in $\alpha 4\beta 2$ nAChRs although the potency decreased 30-fold as indicated by an increase of EC_{50} from 0.012 μM to 0.36 μM . Further, while the 3'-fluoroepibatidine's agonistic potency was similar to that of ($\pm$)-epibatidine (EC_{50} s were 9.8 and 4.8 μM , respectively), the analog evoked a partial (maximal efficacy of 66%), but not full, agonist effect in $\alpha 7$ nAChRs. The assessment of inhibitory effect of the analog on $\alpha 7$ nAChRs registered a development of inhibition at concentrations $>30 \mu M$ with the 50% effect at 225 μM . This suggests that at the concentrations of ~ 10 –30 μM the 3'-fluoro analog induces a full activation of $\alpha 4\beta 2$ nAChRs and approximates its maximal agonist effect in $\alpha 7$ nAChRs without initiating its inhibitory action on the $\alpha 7$ nAChR function. In contrast, the analog induced only 24% activation as maximum at 0.1–100 μM concentration range at $\alpha 3\beta 4$ nAChRs, and inhibited the peak amplitude of ACh(EC_{50})-induced currents with the IC_{50} of $\sim 8.3 \mu M$, suggesting its minor agonist effect on $\alpha 3\beta 4$ nAChRs at the concentrations at which the saturating effect is achieved in $\alpha 4\beta 2$ nAChRs. The dual agonist effect of the 3'-fluoro analog on both $\alpha 4\beta 2$ and $\alpha 7$ nAChRs identified in patch-clamp experiments is consistent with an activity of the analog detected in both [3H]epibatidine (K_i was 25 pM) and [125]iodo-MLA (K_i was 3 nM) binding assays (Carroll et al., 2002). The difference in the potency parameters obtained from the analysis of the functional data and the K_i values are probably due, in part, to a profound desensitization of the nAChRs in binding experiments, however, both functional and binding data demonstrate that $\alpha 4\beta 2$ nAChRs are more sensitive to the 3'-fluoro analog than $\alpha 7$ nAChRs. The functional effect of the analog on $\alpha 4\beta 2$ nAChRs is also consistent with its potent antinociceptive activity in the hot-plate test (ED_{50} was 8 $\mu g/kg$) (Carroll et al., 2002) where nicotine-induced analgesia is mainly mediated by the $\alpha 4\beta 2$ nAChR subtype (Marubio et al., 1999).

Overall, it is important to emphasize that the addition of a 3'-fluoro substitution to epibatidine resulted in an analog with an increased efficacy and markedly improved selectivity for $\alpha 4\beta 2$ versus $\alpha 3\beta 4$ nAChRs while retaining an agonist effect on $\alpha 7$ nAChRs. Previously, functional parameters have been characterized for the 2'-fluoronorchloroepibatidine (Avalos et al., 2002). In contrast to 3'-fluoroepibatidine, the 2'-fluoronorchloroepibatidine exhibited comparable EC_{50} s of 254 and 319 nM and equal efficacies of $\sim 40\%$ in rat versus $\alpha 3\beta 4$ nAChRs expressed in *Xenopus* oocytes. This suggests that the fluoro- substitution at the 3' but not at the 2' position in the pyridine ring improves the selectivity and efficacy of epibatidine for $\alpha 4\beta 2$ nAChRs.

In summary, the dual agonist activity on $\alpha 4\beta 2$ and $\alpha 7$ nAChRs combined with minor activation of $\alpha 3\beta 4$ nAChRs that minimizes its peripheral side effects suggest that the analog of epibatidine with 3'-fluoro substitution may serve as a potential candidate for a treatment of neurological disorders association with a dysfunction of central nAChRs (Gotti et al., 2006a).

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Figure 1.
Structure of epibatidine and 3'-fluoroepibatidine (RTI-31).

Figure 2.

Agonist activity of (±)-epibatidine in $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChRs. A, Representative superimposed currents traces were induced by the application of 1mM ACh and (±)-epibatidine at concentrations that caused about a half activation relative to the ACh response in individual cells expressing indicated nAChR subtype and held at -80 mV. B, Dose-response curves for the peak currents induced by (±)-epibatidine in $\alpha 4\beta 2$ (∇), $\alpha 7$ ($\square$) and $\alpha 3\beta 4$ ($\blacksquare$) nAChRs. Peak currents induced by (±)-epibatidine at different concentrations were normalized to the peak current induced by ACh at 1mM concentration in each cell. Each symbol represents the mean $\pm$ S.E.M. The continuous curves represent a fit to Hill equation. Holding potential was -80 mV.

Figure 3.

Analysis of the agonist activity of the 3'-fluoroepibatidine in $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChRs. A, Dose-response curves for the peak currents induced by the analog in $\alpha 4\beta 2$ (■), $\alpha 7$ (▲) and $\alpha 3\beta 4$ (●) nAChRs. Peak currents induced by the analog at different concentrations were normalized to the peak current induced by ACh at 1 mM concentration in each tested cell. Each symbol represents the mean $\pm$ S.E.M. The continuous curves represent a fit to Hill equation. B, Representative superimposed current traces were induced by the application of 1 mM ACh and the analog at 10 μM concentration in individual cells expressing an indicated nAChR subtype and held at -80 mV. Time constants of the decay (τ) correspond to 26.91 and 38.94 ms in the presence of 1 mM ACh and 10 μM 3'-fluoro analog, respectively, in the representative cell expressing nAChRs. C, Inhibition of 3'-fluoroepibatidine-induced currents in three representative cells expressing $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChRs by DH β E, MLA and mecamylamine, respectively. No response was induced by 3'-fluoroepibatidine in nontransfected SH-EP1 and HEK 293 cells. Holding potential was -80 mV.

Figure 4.

Analysis of the antagonist activity of the 3'-fluoroepibatidine in $\alpha 3\beta 4$ and $\alpha 7$ nAChRs. A, Concentration-response relationships for the analog constructed in $\alpha 3\beta 4$ (■) and $\alpha 7$ (□) nAChRs. The peak amplitude of ACh (EC_{50})-evoked currents was taken in each cell to normalize the peak amplitude of the currents that were evoked in the presence of the analog at different concentrations. The curves were fitted to Hill equation. Symbols and bars represent the mean $\pm$ S.E.M. B, Examples of inhibitory effect of 3'-fluoro substituted analog on ACh (EC_{50})-induced currents in cells expressing $\alpha 7$ (top) and $\alpha 3\beta 4$ (bottom) at concentration close to the determined IC_{50} value. Holding potential in A and B was -80 mV.

Table 1
Efficacy and potency of (±)-epibatidine and 3'-fluoroepibatidine in recombinant $\alpha 4\beta 2$, $\alpha 7$ and $\alpha 3\beta 4$ nAChRs

	$\alpha 4\beta 2$		$\alpha 7$		$\alpha 3\beta 4$	
	$I_{\max}$ (%)	EC_{50} (μM)	$I_{\max}$ (%)	EC_{50} (μM)	$I_{\max}$ (%)	EC_{50} (μM)
(±)-Epibatidine	72	0.012	100	4.8	81	0.027
3'-Fluoroepibatidine	97	0.36	66	9.8	24	n.d.

The values are presented as a mean; n.d., not determined.