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Carbamoylcholine Homologs: Novel and Potent Agonists at Neuronal Nicotinic Acetylcholine Receptors

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ABSTRACT

The classic muscarinic acetylcholine receptor (mAChR) agonist carbamoylcholine (carbachol) does not seem to be the most obvious lead for the development of selective ligands at nicotinic acetylcholine receptors (nAChRs). In the past, however, *N*-methylations of carbachol have provided *N*-methylcarbamoylcholine and *N*,*N*-dimethylcarbamoylcholine (DMCC), which predominantly display nicotinic activity. In this study, 12 homologous analogs of DMCC and its corresponding tertiary amine, *N*,*N*-dimethylcarbamoyl-*N*,*N*-dimethylaminoethanol, were synthesized and their binding affinities to native mAChR and nAChR sites estimated. One of the compounds in the series, 3-*N*,*N*-dimethylaminobutyl-*N*,*N*-dimethylcarbamate (**7**), displayed low nanomolar binding affinity to nAChRs and a 400-fold selectivity for nAChRs over mAChRs. Hence, a new series of compounds was synthesized in which alkyl and aryl groups and different ring systems were introduced in the carbamate moiety of **7**. In a [^{3}H]epibatidine binding assay, the K_i values of **7** and its analogs at rat $\alpha2\beta2$, $\alpha4\beta2$, $\alpha2\beta4$, $\alpha3\beta4$, and $\alpha4\beta4$ nAChRs, stably expressed in mammalian cell lines, ranged from low nanomolar to midmicromolar concentrations, whereas all of the compounds displayed weak binding to an $\alpha7/5\text{-HT}_3$ chimera and to native mAChRs. Compound **7** and its analogs were determined to be agonists at the $\alpha3\beta4$ nAChR subtype. This series includes the most potent and selective nicotinic agonists structurally derived from ACh to date. Furthermore, the compounds are tertiary amines, implying some advantages in terms of bioavailability pertinent to future in vivo pharmacological studies. Finally, observations made in the study hold promising perspectives for future development of ligands selective for specific nAChR subtypes.

The neurotransmitter acetylcholine (ACh) exerts its effects in the central and peripheral nervous systems through two distinct classes of receptors, the muscarinic and nicotinic acetylcholine receptors (mAChRs and nAChRs, respectively). The five cloned mAChR subtypes, m1 to m5, are members of the G-protein-coupled receptor superfamily and mediate their effects through intracellular metabolic cascades (Eglen et al., 2001). In contrast, the nAChRs belong to a superfamily of ligand-gated ion channels that also includes receptors for GABA, glycine, and serotonin (5-HT) (Corringer et al., 2000; Karlin, 2002). The nAChRs are involved in a wide array of physiological functions; over the years, nicotinic ligands have attracted considerable interest as potential therapeutics in the treatment of pain and a number of neurodegenerative and psychiatric disorders (Gotti et al., 1997; Lindstrom, 1997; Arneric and Brioni, 1999; Levin, 2002). Furthermore, nicotine replacement therapy has become the predominant treatment for smoking cessation (Arneric and Brioni, 1999; Levin, 2002).

The nAChR is a transmembrane allosteric protein complex composed of five subunits. So far, 17 nAChR subunits have been cloned and divided into five muscle-type subunits ($\alpha1$, $\beta1$, γ, ϵ, and δ) and 12 neuronal subunits ($\alpha2$-$\alpha10$ and $\beta2$-$\beta4$) (Corringer et al., 2000; Karlin, 2002). The neuronal nAChRs exist either as homomers of $\alpha7$ or $\alpha9$ subunits or as heteromers of various combinations of $\alpha2$ to $\alpha6$ with $\beta2$ to $\beta4$ subunits or $\alpha9$ with $\alpha10$ subunits (Corringer et al., 2000). Subunits $\alpha4$, $\alpha7$, and $\beta2$ are widely expressed in the CNS, whereas the remaining subunits are more differentially expressed. In contrast, $\alpha3$ and $\beta4$ are the predominant subunits in the autonomic ganglia. Thus, the three predominant neuronal nAChRs are the $\alpha4\beta2$, $\alpha3\beta4$* (mostly $\alpha3\alpha5\beta4$ and $\alpha3\beta4$), and $\alpha7$ combinations (Lindstrom, 1997; Corringer et

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ABBREVIATIONS: ACh, acetylcholine; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; 5-HT, 5-hydroxytryptamine; MLA, methyllycaconitine; MCC, *N*-methylcarbamoylcholine; DMCC, *N*,*N*-dimethylcarbamoylcholine; DMCAE, *N*,*N*-dimethylcarbamoyl-*N*,*N*-dimethylaminoethanol; CCh, carbamoylcholine or carbachol; NMS, *N*-methylscopolamine; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; Oxo-M, Oxotremorine-M; FLIPR, fluorometric imaging plate reader; A-85380, 3-[2(*S*)-azetidinylmethoxy] pyridine HCl.

al., 2000). In *Xenopus laevis* oocytes and mammalian cell lines, functional homomeric receptors can be formed by α7 or α9 subunits and by simple heteromeric complexes consisting of an α subunit (α2, α3, and α4) and a β subunit (β2 or β4) in the stoichiometric ratio 2:3. Finally, more diverse receptor complexes composed of several different α2 to α6 and β2 to β4 subunits can be formed (Corringer et al., 2000).

Agonist binding to the muscle nAchR takes place to the amino-terminal regions of the α/γ and α/δ interfaces of the receptor complex, whereas agonist binding to the neuronal nAChRs takes place to the two α/β interfaces of the heteromers and to the five subunit interfaces in the homomers. The crystal structure of an ACh-binding protein from a snail sharing a weak but significant amino acid sequence identity with the amino terminal region of the nAChR has been published (Brejc et al., 2001). The crystal structure has confirmed findings from mutagenesis studies and the highly homologous nature of the agonist binding pockets of the various α/β nAChR combinations (Corringer et al., 2000; Karlin, 2002). This similarity is also reflected in the outcome of medicinal chemistry approaches in the nAChR field. Although a considerable number of compounds have been reported, very few truly subtype-selective nAChR ligands have emerged (Holladay et al., 1997; Arneric and Brioni, 1999; Dwoskin and Crooks, 2001; Romanelli and Gualtieri, 2003). However, the vast majority of these published compounds have not been subjected to a detailed pharmacological characterization at multiple recombinant nAChR combinations.

The majority of nAChR ligands reported so far are derived from substances of natural origin. (*S*)-Nicotine and (±)-epibatidine in particular have been used as leads for new compound series (Holladay et al., 1997; Arneric and Brioni, 1999; Dwoskin and Crooks, 2001; Romanelli and Gualtieri, 2003). In contrast, the endogenous ligand ACh has attracted limited interest as a lead structure, probably reflecting its inherent lack of selectivity between nAChRs and mAChRs and the bioavailability problems associated with the quaternized amino group of ACh. Stabilization of the ester moiety of ACh as a carbamate group has yielded carbamoylcholine (carbachol, CCh), which has been widely used as a mAChR agonist, although it is equipotent as an agonist at neuronal nAChRs (Fig. 1). Introduction of one or two methyl groups at the carbamate nitrogen of carbamoylcholine have yielded *N*-methylcarbamoylcholine (MCC) and *N*,*N*-dimethylcarbamoylcholine (DMCC), respectively, which display nanomolar binding affinities to native nAChRs and a high degree of selectivity for nAChRs versus mAChRs (Fig. 1) (Abood and Grassi, 1986; Punzi et al., 1991; Anderson and Arneric, 1994). This interesting switch in nicotinic/muscarinic selectivity has not been explored to any greater extent. Some years ago, we reported the synthesis of a series of conformationally restricted acyclic and heterocyclic analogs of MCC, DMCC, and the tertiary amine corresponding to DMCC, *N*,*N*-dimethylcarbamoyl-*N*,*N*-dimethylaminoethanol (DMCAE) (Fig. 1) (Søkilde et al., 1996). However, none of these analogs displayed higher binding affinities for native nAChRs than MCC or DMCC.

In the present study, we have synthesized of a series of homologous analogs of DMCC and DMCAE and characterized the compounds pharmacologically at native mAChRs as well as native and recombinant neuronal nAChRs.

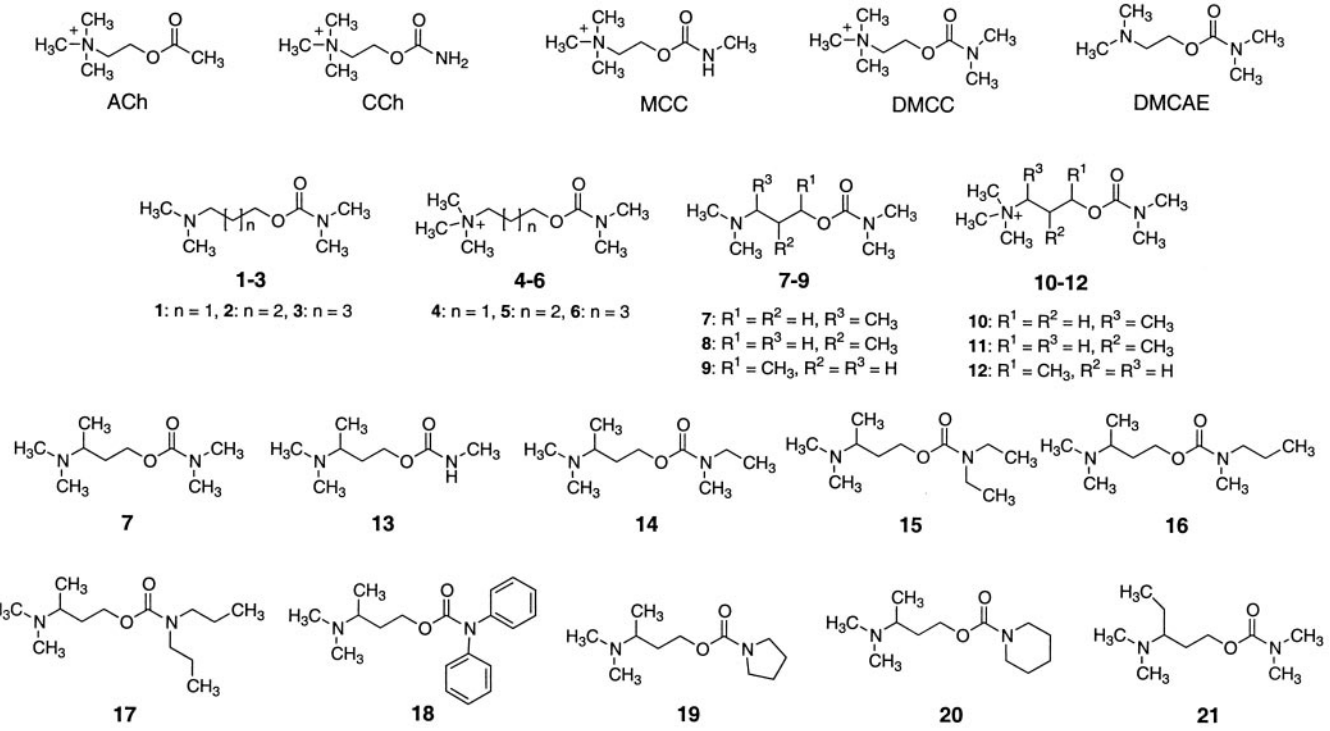

Fig. 1. Chemical structures of the compounds characterized pharmacologically in this study. The structures of ACh, CCh, MCC, DMCC, and DMCAE are given for comparison. Although the tertiary amines DMCAE, **1**-**3**, **7** to **9**, and **13** to **21** are almost completely but reversibly protonated at physiological pH, they are depicted in the nonprotonated form. The compounds **1** to **12** were characterized in binding assays to native mAChRs and nAChRs, whereas compounds **13** to **21** together with **7** were characterized in greater detail at numerous recombinant nAChRs. Compounds **7** to **21** were tested as racemic mixtures.

Materials and Methods

Materials. Culture media, serum, antibiotics, and buffers for cell culture were obtained from Invitrogen (Groningen, The Netherlands). G418 sulfate, blasticidin S HCl, and pCDNA3.1 and pCDNA6-V5/His vectors were also obtained from Invitrogen. [^{3}H]Oxotremorine-M ([^{3}H]Oxo-M), [^{3}H](±)-epibatidine ([^{3}H]epibatidine), and *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) were purchased from PerkinElmer Life Sciences (Zaventem, Belgium), (*S*)-[^{3}H]nicotine from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), and [^{3}H]methyllycaconitine ([^{3}H]MLA) from Tocris (Bristol, UK). GF/B and GF/C filters were obtained from Whatman Paper Ltd. (Gaithersburg, MD). ACh, (*S*)-nicotine tartrate, CCh, and MCC were obtained from Sigma-RBI (St. Louis, MO), whereas MLA, (±)-epibatidine, (−)-cytisine, and lobeline were obtained from Tocris (Bristol, UK).

The cDNAs encoding the rat α2, α4, α7, β2, and β4 nAChR subunits were kind gifts from Dr. James W. Patrick (Baylor College of Medicine, Houston, TX). Dr. David J. Julius (University of California, San Francisco, CA) kindly provided us with cDNA for the murine 5-HT$_{3A}$ receptor. A human embryonic kidney HEK293 cell line stably expressing the rat α3β4 nAChR (the cell line KXα3β4R2) was a generous gift from Drs. Ken Kellar and Yingxian Xiao (Georgetown University School of Medicine, Washington DC) (Xiao et al., 1998). A HEK293 cell line stably expressing rat α4β2 nAChR was kindly provided by Dr. Joe Henry Steinbach (Washington University School of Medicine, St. Louis, MO) (Sabey et al., 1999). The tsA cells were a gift from Dr. Penelope S. V. Jones (University of California, San Diego, CA).

Chemistry. The target compounds were all synthesized using the same approach as outlined in Fig. 2 via the appropriate amino alcohols. The methyl substituted analogs **7** to **9** were synthesized by addition of dimethylamine to the appropriate α,β-unsaturated esters followed by reduction to the corresponding amino alcohol using either lithium aluminum hydride or catalytic hydrogenation. Introduction of the carbamoyl group was performed using sodium hydride followed by the appropriate alkylated carbamoyl chloride. Most of the tertiary amines, **1** to **3**, **7** to **9**, and **13** to **20** were isolated as fumarate salts. The corresponding quaternized analogs **4** to **6** and **10** to **12** were prepared from the tertiary amines by alkylation with methyl iodide.

All of the compounds were characterized by ^{1}H-NMR. Melting points were determined in capillary tubes and are uncorrected. Analytical thin-layer chromatography was carried out using Merck silica gel 60 F254 plates. ^{1}H-NMR spectra were recorded on a Bruker AC-200 F spectrometer using $CDCl_3$ solutions and tetramethyl silane as an internal standard or in D_2O solutions using acetonitrile as an internal standard. Elemental analyses were carried out and analyses are within ± 0.4% of the theoretical values. Detailed experimental procedures and spectroscopic and analytical data of the compounds are available by enquiry to the author of correspondence.

Molecular Biology. The β2, β4, and α7 subunits were subcloned from their original pCDNAI/Neo vector into pCDNA3.1 using *Hin*dIII/*Xba*I (β2 and α7) or *Hin*dIII/*Xho*I (β4) as unique restriction enzymes. The α2 and α4 were subcloned from pCDNAI/Neo to pCDNA6-V5/His using *Hin*dIII and *Xba*I as unique restriction enzymes.

For the construction of the α7/5-HT$_3$ chimera, a silent *Bsp*EI site was introduced in 5-HT$_{3A}$-pCDNA3.1 (covering nucleotides 728 to 733), and a *Bsp*EI site was introduced in the corresponding site region of α7-pCDNA3.1 (covering nucleotides 677–682). Subsequently, the cDNA encoding the amino-terminal part of α7 was subcloned into 5-HT$_{3A}$-pCDNA3.1 using *Hin*dIII and *Bsp*EI as restriction enzymes. The resulting α7/5-HT$_3$ chimera consisted of Met^1-Val^{224} of α7 and Ile^{242}-Ser^{483} of 5-HT$_{3A}$ and is analogous to the V201 α7/5-HT$_3$ chimera characterized in a previous study (Eiselé et al., 1993). Amplified DNAs were sequenced on an ABI Prism 310 using Big Dye terminator cycle sequencing kit (Perkin-Elmer Life Sciences, Warrington, UK).

Production of Stable nAChR Cell Lines. For the stable expression of α2β2, α2β4, and α4β4 nAChRs in tsA cells [a transformed HEK293 cell line (Chahine et al., 1994)], the cells were maintained at 37°C in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% calf serum. The cells were transfected with the respective α/β combinations, using Polyfect as a DNA carrier according to the manufacturer's protocol (QIAGEN, Hilden, Germany) and maintained for 2 to 3 weeks in selection medium containing 2 mg/ml G418 and 1 μg/ml blasticidin. Antibiotic-resistant colonies were isolated and maintained in the presence of the selection medium.

Cell Culture. All cell lines were maintained at 37°C in a humidified 5% CO_2 incubator in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% calf serum. HEK293 cells stably expressing α4β2 and α3β4 nAChRs were cultured in medium containing G418 (0.5 mg/ml and 0.7 mg/ml, respectively). The tsA cells stably expressing α2β2, α2β4, and α4β4 nAChRs were grown in medium supplemented with G418 (1 mg/ml) and blasticidin (1 μg/ml). The α7/5-HT$_3$ chimera was transiently expressed in tsA cells cultured in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% calf serum. Cells (2×10^6) were split

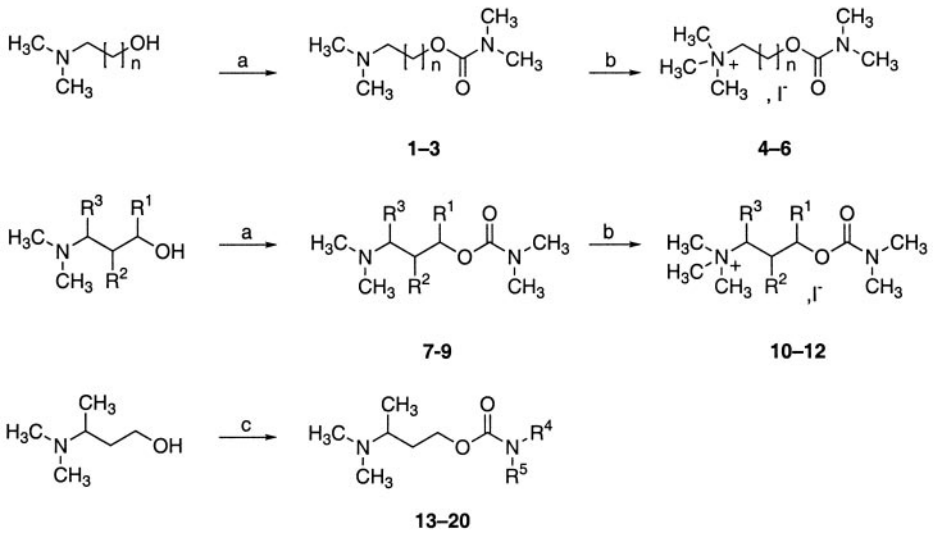

Fig. 2. Synthesis of the target compounds. Reagents: (a) NaH, then *N*,*N*-dimethylcarbamoyl chloride, toluene; (b) MeI and EtOH or acetone; (c) NaH, then *N*,*N*-R^{4}R^5-carbamoyl chloride, toluene. The general structures refer to the compounds presented in Fig. 1.

into a 15-cm tissue culture plate a day before transfection and transfected with 10 μg α7/5-HT$_3$-pCDNA3.1 using Polyfect as a DNA carrier. The day after the transfection the medium was changed; on the following day, [^{3}H]MLA binding was performed.

[^{3}H]Oxo-M and (*S*)-[^{3}H]Nicotine Binding Assays. IC_{50} values for the compounds at native mAChRs and nAChRs were estimated in binding assays as described previously (Søkilde et al., 1996). In the [^{3}H]Oxo-M binding assay, rat brains were homogenized in 100 volumes (w/v) of 10 mM sodium potassium phosphate buffer, pH 7.4, and diluted 1:10 with the same buffer. Membranes (5 mg) were incubated with 0.2 nM [^{3}H]Oxo-M in the absence or presence of test compound in a total reaction volume of 1.5 ml at 30°C for 40 min. Incubation was stopped by adding 5 ml of ice-cold buffer, followed by rapid filtration through Whatman GF/B filters presoaked in 0.1% polyethylenimine using a Brandel cell-harvester, and two subsequent washes with 5 ml of ice-cold buffer. Nonspecific binding was determined using 10 μM atropine.

In the (*S*)-[^{3}H]nicotine binding assay, rat brains were homogenized in 10 vol (w/v) of homogenization buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 120 mM NaCl, 2 mM EDTA, 20 mM HEPES, and 5 mM iodoacetamide, pH 7.4). The homogenate was centrifuged (50,000*g*, 20 min, 4°C), and the pellet resuspended in 10 volumes of assay buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 120 mM NaCl, 20 mM HEPES, 1 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4). Membranes (0.1 mg) were incubated with 5 nM (*S*)-[^{3}H]nicotine in the absence or presence of test compound in a total reaction volume of 0.6 ml on ice for 60 min. Incubation was stopped by adding 5 ml of ice-cold 50 mM sodium potassium phosphate buffer, pH 7.4, followed by rapid filtration through Whatman GF/B filters presoaked in 0.1% polyethylenimine using a Brandel cell-harvester, and three subsequent washes with 5 ml of ice-cold sodium potassium phosphate buffer. Nonspecific binding was determined using 0.5 mM (*S*)-nicotine tartrate.

The filters from the [^{3}H]Oxo-M and (*S*)-[^{3}H]nicotine binding experiments were dried, and the amount of bound radioactivity was determined in a scintillation counter. On basis of preliminary experiments, concentrations of the test compounds resulting in 25 to 75% inhibitions of radioligand binding were chosen for estimation of IC_{50} values. Each compound was tested in triplicate at three different concentrations, and each displacement experiment was performed at least four times.

[^{3}H]Epibatidine Binding. Binding was performed after a protocol slightly modified from that used in a previous study (Parker et al., 1998). Cells were harvested at 80 to 90% confluence and scraped into assay buffer (140 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM Mg_2SO_4, and 25 mM HEPES, pH 7.4), homogenized with a Polytron homogenizer for 10 s, and centrifuged for 20 min at 50,000*g*. Cell pellets were resuspended in fresh assay buffer, homogenized, and centrifuged at 50,000*g* for another 20 min. Cells were resuspended in assay buffer, and protein concentrations were measured using the Bradford protein assay with bovine serum albumin as the standard (Bio-Rad, Hercules, CA). The total amount of protein in each reaction was 5 to 15 μg for the $\alpha2\beta2$, $\alpha4\beta2$, $\alpha2\beta4$ and $\alpha4\beta4$ cell lines, whereas amounts below 1 μg were used for $\alpha3\beta4$-HEK293.

In the saturation binding experiments, cell membranes were incubated with 12 concentrations of [^{3}H]epibatidine (0.01 pM to 6 nM) at room temperature for 4 h while shaking. Reaction volumes varied depending on the radioligand concentration used: for concentrations above 300 pM, the total volume was 0.5 ml; for concentrations between 50 and 300 pM, the total volume was 2 ml; and for concentrations below 50 pM, volumes of 4 ml were used. After this procedure, the fraction of specifically bound ligand was always <10% of the total ligand, and in most cases it was <2%. Nonspecific binding was determined in parallel reactions containing 5 mM (*S*)-nicotine.

In the competition binding experiments, cell membranes were incubated with [^{3}H]epibatidine at concentrations of 100 to 200 pM (β2-containing nAChRs) or 500 to 700 pM (β4-containing nAChRs) in the presence of various concentrations of compounds. The total reaction volume was 0.5 ml, and the reactions were incubated for 4 h at room temperature while shaking. Whatman GF/B filters were presoaked for 1 h in a 0.2% polyethylenimine solution, and binding was terminated by filtration through these filters using a 48-well Brandel cell harvester and four washes with 4 ml of ice-cold isotonic NaCl solution. After this, the filters were dried, 3 ml of Opti-Fluor (PerkinElmer Life Sciences was added, and the amount of bound radioactivity was determined in a scintillation counter. The binding experiments were performed in duplicate at least three times for each compound.

[^{3}H]MLA Binding. In the binding experiments with α7/5-HT$_3$ transfected tsA cells, a 20-cm tissue culture plate of cells were scraped into homogenization buffer (50 mM Tris-HCl, pH 7.2), homogenized for 10 s in 30 ml of homogenization buffer, and centrifuged for 20 min at 50,000*g*. In the binding experiments using rat brain membranes, membrane preparation was performed using a method described previously (Ransom and Stec, 1988). On the day of experiments, frozen homogenates were quickly thawed, homogenized for 10 s in 30 ml of homogenization buffer (50 mM Tris-HCl, pH 7.2), and centrifuged for 20 min at 50,000*g*. The resulting pellets of tsA cells or rat brain membranes were homogenized in 30 ml of homogenization buffer and centrifuged again. This step was performed twice. Then pellets were resuspended in phosphate-buffered saline, and protein concentrations were determined as described previously. The protocol used for the binding assay was slightly modified from that of a previous study (Davies et al., 1999). Protein (5 to 15 μg) from α7/5-HT$_3$-transfected tsA cells or 50 to 100 μg of protein from rat brain membranes was used for each reaction, and the total assay volume was 2 ml. In the saturation binding experiments, rat brain homogenate or tsA cells were incubated with 12 different concentrations of [^{3}H]MLA (0.03 to 20 nM) in the absence or presence of 5 mM (*S*)-nicotine (total and nonspecific binding, respectively). In the competition binding experiments, 8 to 12 different concentrations of the ligands and 1 nM [^{3}H]MLA were used. In this way, the percentage of radioligand bound was <5% of the free radioligand in all experiments.

The assay mixtures were incubated for 2.5 h at room temperature while shaking. GF/B filters were presoaked for 1 h in a 0.2% polyethylenimine solution, and binding was terminated by filtration through these filters using a 48-well Brandel cell harvester and four washes with 4 ml of ice-cold isotonic NaCl solution. After this, the filters were dried, 3 ml of Opti-Fluor were added, and the amount of bound radioactivity was determined in a scintillation counter. The binding experiments were performed in duplicate at least three times for each compound.

[^{3}H]NMS Binding. The [^{3}H]NMS binding protocol used was slightly modified from that used in a previous study (Dörje et al., 1991). Brain synaptic membranes from male Sprague-Dawley rat were used, and tissue preparation was performed as described previously (Ransom and Stec, 1988). On the day of assays, the membrane preparation was quickly thawed, suspended in assay buffer (25 mM sodium phosphate, pH 7.4, supplemented with 5 mM $MgCl_2$), homogenized, and centrifuged at 20,000*g* at 4°C for 30 min. This step was repeated once, after which the pellet was resuspended in assay buffer, and protein concentrations were measured as described previously. Membranes (50–100 μg of protein) were incubated on ice for 6 h in the presence of 5 pM [^{3}H]NMS and eight different concentrations of the various compounds in a total assay volume of 1 ml. Nonspecific binding was determined in the presence of 10 μM atropine. GF/C filters were presoaked for 1 h in a 0.2% polyethylenimine solution, and binding was terminated by filtration through these filters using a 48-well Brandel cell harvester and three washes with 5 ml of ice-cold isotonic NaCl solution. After this, the filters were dried, 3 ml of Opti-Fluor was added, and the amount of bound radioactivity was determined in a scintillation counter. The binding experiments were performed in duplicate at least three times for most of the compounds and two times for a few of them.

Functional Characterization Using the FLIPR Membrane Potential Assay. The compounds were characterized functionally at the $\alpha3\beta4$-HEK293 cell line (Xiao et al., 1998) using the FLIPR membrane potential assay kit according to the manufacturer's protocol (Molecular Devices, Crawley, UK). Cells were split into poly-D-lysine–coated black 96-well plates (BD Biosciences, Palo Alto, CA) in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), 10% calf serum, and 0.7 mg/ml G418. Sixteen to 24 h later, the medium was aspirated, and 50 μl of fresh medium supplemented with 2 μM atropine was added to each well. Then, 50 μl of loading buffer (dye dissolved in Hanks' buffered saline solution supplemented with 20 mM HEPES, pH 7.4) was added to each well, and the plate was incubated at 37°C in a humidified 5% CO_2 incubator for 30 min. Thus, the final concentration of atropine in the assay was 1 μM. The plate was assayed in a NOVOstar plate reader (BMG Labtechnologies, Offenburg, Germany) measuring emission at 560 nm caused by excitation at 530 nm. Data points were measured at room temperature a total of 77 times during 1 min. Three of the recordings were done before application of 33 μl of agonist solution (agonists were diluted in Hanks' buffered saline solution supplemented with 20 mM HEPES, pH 7.4). The experiments were performed in duplicate at least three times for each compound. The concentration-response curve for each of the compounds was constructed based on the maximal responses obtained for the eight different concentrations used.

Data Analysis. Data from the saturation binding experiments were fitted to a single-site ligand binding model, and dissociation constants (K_D) and maximum binding values (B_{max}) were determined by nonlinear regression. Data from the competition binding experiments were fitted to the equation %Bound = 100% Bound/(1 + ([L]/IC_{50})^{n_H}), and K_i values were determined using the equation K_i = IC_{50}/(1 + [L]/K_D), where [L] is the radioligand concentration, n_H is the Hill coefficient, and K_D is the dissociation constant. Data from the functional experiments were fitted to the simple mass equation: R = R_{basal} + [R_{max}/(1 + (EC_{50}/[A]^{n_H}))], where [A] is the concentration of agonist, n_H is the Hill coefficient, and R is the response. Curves were generated by nonweighted least-squares fits using the program KaleidaGraph 3.08 (Synergy Software, Reading, PA).

Results

Pharmacological Screening of Compounds 1 to 12 at Native mAChRs and nAChRs. To approximate the affinities of compounds **1** to **12** at native mAChRs and nAChRs, the compounds were characterized in [^{3}H]Oxo-M and (*S*)-[^{3}H]nicotine binding assays using rat brain tissue. Because the concentrations of tracer used in the two assays were similar to [(*S*)-[^{3}H]nicotine] or significantly lower than ([^{3}H]Oxo-M) the reported K_D values of the radioligands, the IC_{50} values obtained for compounds in this assay can be assumed to be very similar to the K_i values. The estimated K_i values of the compounds in the two assays together with the nAChR/mAChR affinity ratios for the compounds are given in Table 1. Extension of DMCAE and DMCC with one methylene group led to compounds **1** and **4** with very different pharmacological profiles. Compound **4**, the one-carbon homolog of DMCC, exhibited a 400-fold lower nicotinic affinity than its parent compound, whereas compound **1** displayed a 10-fold higher binding affinity than DMCAE (Fig. 1 and Table 1). Further extension of the backbone of **1** with one or two additional methylene groups to give **2** and **3** resulted in dramatically decreased binding affinities for the native nAChRs. Further extension of the backbone of compound **4** led to more moderate decreases in nicotinic binding affinity (compounds **5** and **6** in Table 1). The binding affinities of compounds **1** to **3** and **4** to **6** for native mAChRs were comparable with or slightly higher than those of DMCAE and DMCC, respectively (Table 1).

To study the pharmacological effects of methyl substitutions at each of the three backbone carbon atoms of compounds **1** and **4**, compounds **7** to **9** and the corresponding quaternized amines **10** to **12** were synthesized (Fig. 1). The introduction of a methyl group at C-1 (R^1) of **1** and **4** to give compounds **9** and **12**, respectively, essentially eliminated binding of the compounds to native nAChRs (Fig. 1 and Table 1). In contrast, the binding affinities of **9** and **12** to native mAChR sites were similar to those of **1** and **4**, respectively. A methyl-substitution at C-2 (R^2) of **1** and **4** to give compounds **8** and **11**, respectively, also decreased the binding affinities for nAChRs as well as for mAChRs (Fig. 1 and Table 1). Introduction of a methyl group at C-3 (R^3) of **4** to give compound **10** resulted in a minor decrease in affinity for mAChR as well as nAChR sites. In contrast, the corresponding tertiary amine **7** exhibited a 70-fold higher affinity for nAChRs than its parent compound **1** (Fig. 1 and Table 1). Furthermore, compound **7** displayed 400-fold selectivity for native nAChRs over native mAChRs (Table 1). To investigate this new lead further, a new series of compounds were synthesized (compounds **13-21** in Fig. 1). These compounds and **7** were characterized pharmacologically in greater detail at recombinant nAChRs expressed in mammalian cell lines.

Competition for [^{3}H]Epibatidine Binding to Heteromeric nAChRs. Before the characterization of compounds **7** and **13** to **21**, we wanted to verify that the pharmacological characteristics of the five α/β nAChR heteromers stably expressed in tsA or HEK293 cells were similar in our [^{3}H]epibatidine binding assay compared with those obtained in previous studies. Hence, K_D and B_{max} values for the radioligand and binding affinities for five standard nicotinic ligands for the various receptor combinations were determined (Table 2 and Fig. 3). The K_D values of [^{3}H]epibatidine at $\alpha2\beta2$, $\alpha4\beta2$, $\alpha2\beta4$, $\alpha3\beta4$, and $\alpha4\beta4$ were all in excellent agreement with those previously obtained at recombinant rat and human

TABLE 1

Estimated binding affinities of compounds **1** to **12** at native nAChRs and mAChRs

The (*S*)-[^{3}H]nicotine and [^{3}H]Oxo-M binding to rat brain tissue were performed as described under *Materials and Methods*. The N/M ratio is defined as K_i ([^{3}H]Oxo-M)/K_i [(*S*)-[^{3}H]nicotine]. The first nine compounds listed in the table are quaternized amines; the others are tertiary amines. Data for CCh, MCC, DMCC, and DMCAE are from Søkilde et al. (1996).

Compound	Receptor Binding (K_i)		
	(*S*)-[^{3}H]Nicotine	[^{3}H]Oxo-M	N/M Ratio
	nM	*nM*	
CCh	750	5	0.007
MCC	23	150	7
DMCC	20	1,200	60
4	7,400	870	0.1
5	70,000	390	0.006
6	47,000	910	0.02
10	48,000	1,800	0.04
11	48,000	9,800	0.2
12	>100,000	1,400	<0.01
DMCAE	5,700	18,000	3
1	630	4,700	8
2	>100,000	2,900	<0.03
3	>100,000	1,300	<0.01
7	9	3,600	400
8	>100,000	10,000	<0.1
9	20,000	19,000	1

nAChRs expressed in *X. laevis* oocytes and mammalian cells (Chavez-Noriega et al., 1997; Parker et al., 1998; Stauderman et al., 1998; Xiao et al., 1998). Furthermore, the B_{max} values of the $\alpha3\beta4$- and $\alpha4\beta2$-HEK293 cell lines were in agreement with the previously reported values for these cell lines (Xiao et al., 1998; Sabey et al., 1999). Finally, the K_i and n_H values of ACh, (*S*)-nicotine, (−)-cytisine, (±)-epibatidine, and lobeline were in agreement with previously reported values at recombinant rat nAChRs (Table 2) (Parker et al., 1998; Xiao et al., 1998). The five standard ligands displayed binding affinities for $\alpha4\beta2$ similar to or slightly lower than those for $\alpha2\beta2$, 10- to 30-fold lower affinities for $\alpha2\beta4$ and $\alpha4\beta4$, and 100-fold lower affinities for $\alpha3\beta4$.

In Fig. 4, displacement curves for a selection of the carbamoylcholine homologs at the five heteromeric nAChRs are depicted. The K_i values for the entire series (compounds **7** and **13-21)** are given in Table 2. The K_i values ranged from subnanomolar to midmicromolar concentrations. The Hill coefficients of all the compounds were around or slightly below unity (data not shown).

Competition for [^{3}H]MLA Binding at α7/5-HT$_3$. It is well documented that the $\alpha7$ subunit is unable to reach the cell surface when transiently expressed in mammalian cells (Dineley and Patrick, 2000). In contrast, α7/5-HT$_3$ constructs such as the V201 chimera (similar to the α7/5-HT$_3$ chimera used in this study) have been shown to reach the cell surface and display ^{125}I-α-bungarotoxin binding characteristics comparable with those of the wild-type $\alpha7$ receptor (Eiselé et al.,

TABLE 2

Binding characteristics at heteromeric nAChRs

The K_D and B_{max} values of [^{3}H]epibatidine at the rat α/β nAChRs determined in saturation binding experiments are given in italics. The $\alpha4\beta2$ and $\alpha3\beta4$ nAChRs were stably expressed in HEK 293 cells, and the $\alpha2\beta2$, $\alpha2\beta4$, and $\alpha4\beta4$ nAChRs were stably expressed in tsA cells. Binding experiments were performed as described under *Materials and Methods*. Each experiment was performed in duplicate at least three times.

Compound	K_i (pK_i ± S.E.M.)				
	$\alpha2\beta2$	$\alpha4\beta2$	$\alpha2\beta4$	$\alpha3\beta4$	$\alpha4\beta4$
			nM		
[^{3}H]Epibatidine					
K_D (pM)	*21 ± 4.7*	*41 ± 6.7*	*90 ± 8.7*	*360 ± 35*	*101 ± 7.8*
B_{max} (pmol/mg)	*0.43 ± 0.04*	*1.4 ± 0.12*	*1.4 ± 0.23*	*7.9 ± 0.35*	*0.91 ± 0.12*
ACh	4.2 (8.38 ± 0.05)	33 (7.48 ± 0.11)	110 (6.97 ± 0.05)	620 (6.21 ± 0.08)	58 (7.23 ± 0.12)
(*S*)-Nicotine	3.6 (8.44 ± 0.07)	8.3 (8.08 ± 0.12)	83 (7.08 ± 0.01)	290 (6.54 ± 0.05)	74 (7.13 ± 0.11)
(−)-Cytisine	0.54 (9.26 ± 0.07)	1.3 (8.89 ± 0.24)	4.6 (8.34 ± 0.01)	130 (6.87 ± 0.03)	6.8 (8.17 ± 0.36)
(±)-Epibatidine	0.023 (10.64 ± 0.08)	0.046 (10.34 ± 0.14)	0.091 (10.04 ± 0.12)	0.46 (9.34 ± 0.11)	0.072 (10.01 ± 0.14)
Lobeline	1.6 (8.79 ± 0.05)	2.2 (8.65 ± 0.22)	280 (6.56 ± 0.13)	480 (6.32 ± 0.13)	120 (6.94 ± 0.21)
MCC	24 (7.62 ± 0.09)	28 (7.55 ± 0.19)	310 (6.51 ± 0.09)	2,700 (5.57 ± 0.10)	620 (6.21 ± 0.06)
7	9.2 (8.04 ± 0.12)	17 (7.77 ± 0.04)	100 (6.99 ± 0.02)	370 (6.43 ± 0.04)	66 (7.18 ± 0.09)
13	3.6 (8.44 ± 0.08)	13 (7.89 ± 0.21)	530 (6.28 ± 0.07)	10,000 (4.98 ± 0.07)	2,600 (5.58 ± 0.08)
14	4.2 (8.38 ± 0.09)	5.6 (8.25 ± 0.02)	21 (7.68 ± 0.06)	62 (7.21 ± 0.06)	13 (7.90 ± 0.08)
15	8.2 (8.09 ± 0.09)	12 (7.91 ± 0.09)	23 (7.63 ± 0.03)	140 (6.85 ± 0.04)	31 (7.51 ± 0.01)
16	35 (7.46 ± 0.09)	62 (7.21 ± 0.06)	300 (6.53 ± 0.12)	5,000 (5.30 ± 0.03)	1,800 (5.74 ± 0.01)
17	2,900 (5.53 ± 0.08)	1,513 (5.82 ± 0.18)	7,200 (5.14 ± 0.09)	86,000 (4.06 ± 0.05)	2,000 (5.71 ± 0.11)
18	35,000 (4.45 ± 0.11)	52,000 (4.28 ± 0.13)	54,000 (4.26 ± 0.10)	81,000 (4.09 ± 0.08)	42,000 (4.37 ± 0.05)
19	61 (7.21 ± 0.14)	130 (6.90 ± 0.05)	1,200 (5.89 ± 0.03)	19,000 (4.71 ± 0.07)	3,300 (5.49 ± 0.01)
20	43 (7.37 ± 0.03)	130 (6.90 ± 0.08)	260 (6.58 ± 0.06)	1,500 (5.81 ± 0.06)	240 (6.63 ± 0.06)
21	56 (7.25 ± 0.08)	140 (6.85 ± 0.18)	210 (6.68 ± 0.04)	1,900 (5.72 ± 0.06)	340 (6.47 ± 0.11)

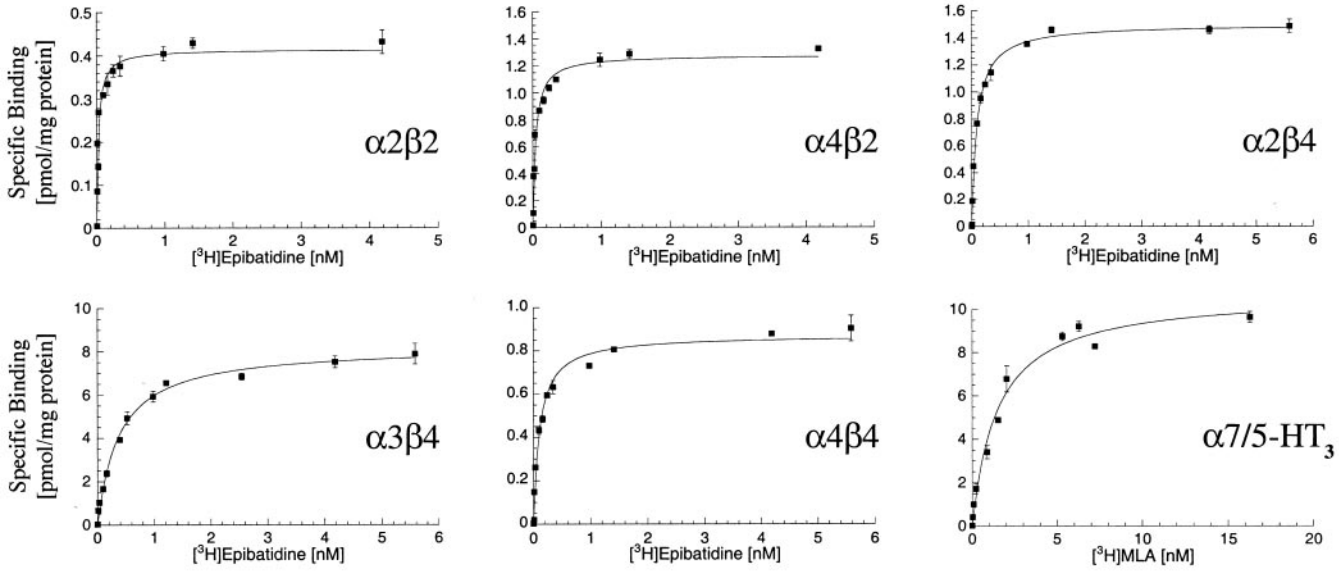

Fig. 3. Saturation binding of [^{3}H]epibatidine to rat $\alpha2\beta2$, $\alpha4\beta2$, $\alpha2\beta4$, $\alpha3\beta4$, and $\alpha4\beta4$ nAChRs stably expressed in HEK293 or tsA cells and of [^{3}H]MLA to α7/5-HT$_3$ transiently expressed in tsA cells. The binding experiments were performed as described under *Materials and Methods*. Data shown are from individual experiments.

1993; Dineley and Patrick, 2000). However, these chimera have not been characterized in a [^{3}H]MLA binding assay. Hence, to verify that radioligand binding to the recombinant chimera did indeed reflect binding to native α7 receptors, we performed [^{3}H]MLA binding experiments using both α7/5-HT$_3$ transfected tsA cells and rat brain membranes. The K_D value of [^{3}H]MLA and the K_i values for ACh, (*S*)-nicotine, (±)-epibatidine, (−)-cytisine, lobeline, and MLA obtained using rat brain tissue were very similar to those obtained with α7/5-HT$_3$-transfected tsA cells (Table 3). Furthermore, the K_D and K_i values at α7/5-HT$_3$ were in excellent agreement with those obtained in previous studies of [^{3}H]MLA binding to native rat and invertebrate α7 nAChRs (Davies et al., 1999; Lind et al., 2001). All of the carbamoylcholine homologs under study displayed very weak binding properties to the α7/5-HT$_3$ chimera (Fig. 4 and Table 3).

Functional Characterization of Compounds at α3β4-HEK293. The functional characterization of the compounds at the α3β4 nAChRs was performed using the FLIPR membrane potential assay (Molecular Devices). The presence of 1 μM atropine in the assay was due to the endogenous mAChR in HEK293 cells. Atropine has recently been reported to inhibit α3β4 nAChR function at micromolar concentrations (Parker et al., 2003). However, the pharmacological characteristics (potencies, Hill slopes, and maximal responses) of the nAChR-specific agonists (*S*)-nicotine, (±)-epibatidine, and (−)-cytisine at the α3β4-HEK293 cell line in the presence of 1 μM atropine were not significantly different from those obtained without atropine (data not shown).

Exposure of α3β4-HEK293 to nicotinic agonists elicited a solid concentration-dependent increase in fluorescent intensity. The data obtained using this assay were highly reproducible (Fig. 5A). The potencies of standard agonists ACh, (*S*)-nicotine, (−)-cytisine, and (±)-epibatidine at α3β4 were 2- to 4-fold higher than those previously reported for rat and human α3β4 receptors expressed in *X. laevis* oocytes or mammalian cells and assayed by means of electrophysiology in fluorescence-based Fura-2/$[Ca^{2+}]_i$ assays or in $^{86}Rb^+$ assays (Table 4) (Covernton et al., 1994; Chavez-Noriega et al., 1997; Stauderman et al., 1998; Xiao et al., 1998). However, the rank order of agonist potencies in the assay was in excellent agreement with these studies [(±)-epibatidine ≫ (−)-cytisine ≥ (*S*)-nicotine > ACh]. The Hill slopes for the agonists were all significantly higher than unity, which is in agreement with previous studies (Table 4) (Chavez-Noriega et al., 1997; Stauderman et al., 1998; Xiao et al., 1998). In a very recent study, KXα3β4R2 and other nAchR cell lines have been characterized pharmacologically in the FLIPR membrane potential assay also used in this study (Fitch et al., 2003). In agreement with our findings, the authors found agonist potencies at KXα3β4R2 to be higher than those obtained from $[Ca^{2+}]_i$ measurements (Fitch et al., 2003). However, in contrast to studies of nAChRs expressed in *X. laevis* oocytes and mammalian cells and assayed by electrophysiology or $[Ca^{2+}]_i$ measurements (Chavez-Noriega et al., 1997; Stauderman et al., 1998), the FLIPR membrane potential assay seemed to be unable to discriminate between full and partial agonists at the nAchRs (Fitch et al., 2003). In agreement with this, all agonists characterized in this study displayed maximal responses similar to that of ACh (data not shown).

The functional characteristics of compounds **7** and **13** to **21** at α3β4 are given in Table 4 and in Fig. 5B. All compounds were agonists with potencies in the micromolar range and Hill coefficients higher than unity, except compound **18**, which displayed millimolar potency and a Hill coefficient below unity. The most potent compounds in the series were **14** and **15**, which were slightly more potent than (−)-cytisine and (*S*)-nicotine and 15-fold more potent than MCC (Table 4). There was a clear correlation between the affinities obtained for the compounds in [^{3}H]epibatidine binding to α3β4 and their potencies at the receptor (Fig. 5C). Correlation analysis

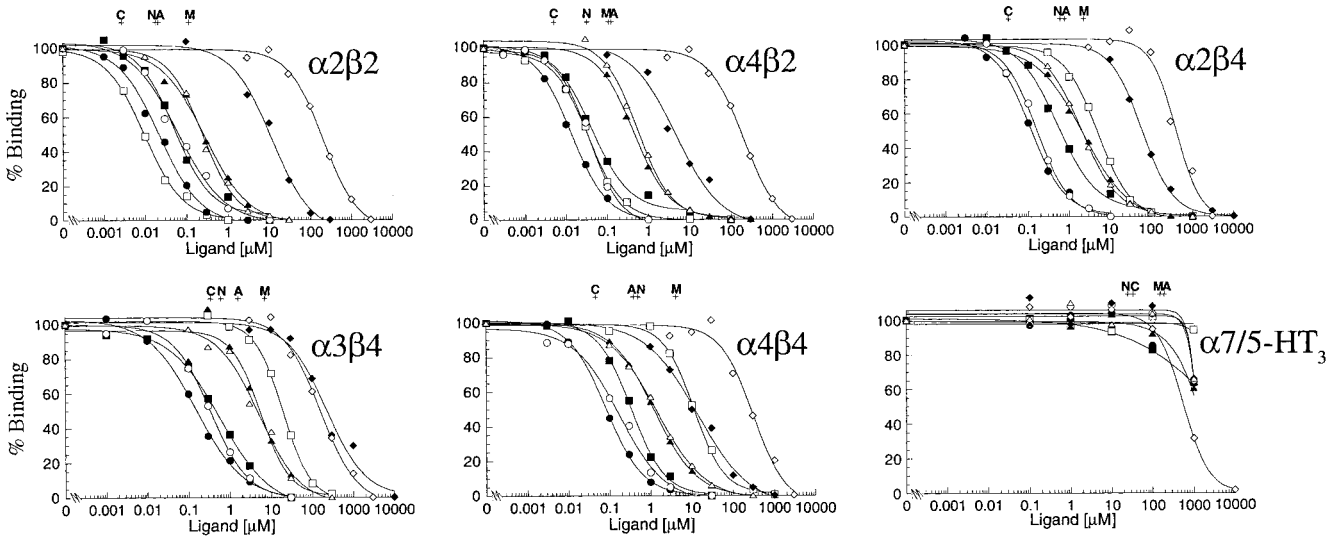

Fig. 4. Competition for [^{3}H]epibatidine or [^{3}H]MLA binding by selected carbamoylcholine homologs. Concentration-response (given as percentage of specific binding) curves for compounds **7** (■), **13** (□), **14** (●), **15** (○), **17** (♦), **18** (◇), **20** (▲), and **21** (△) at α2β2, α4β2, α2β4, α3β4, α4β4, and the α7/5-HT$_3$ chimera expressed in HEK293 or tsA cells. For comparison, the IC_{50} values of ACh (A), (*S*)-nicotine (N), (−)-cytisine (C), and MCC (M) are depicted above the concentration-response curves. The binding experiments were performed as described under *Materials and Methods*. Homogenates of cells expressing the various nAChRs were incubated with 100 to 200 pM [^{3}H]epibatidine (β2-containing nAChRs), 500 to 700 pM [^{3}H]epibatidine (β4-containing nAChRs), or 1 nM [^{3}H]MLA (α7/5-HT$_3$) in the presence of various concentrations of ligands at room temperature for 4 h (α/β nAChRs) or 2.5 h (α7/5-HT$_3$) while shaking. Data shown are from individual experiments. Error bars have been omitted for clarity.

between affinities and potencies of all compounds in Table 4 yielded an r^2 value of 0.95. The r^2 value for compounds **7** and **13** to **21** alone was also 0.95 (data not shown).

Competition for [^{3}H]NMS Binding at Native mAChRs. To determine the binding affinities of compounds **7** and **13** to **21** to native mAChRs, we performed [^{3}H]NMS competition binding to rat brain membranes. Because the K_D values of [^{3}H]NMS for all five mAChR subtypes are significant higher than the 5 pM used as tracer concentration in this assay (Dörje et al., 1991), it is reasonable to assume that IC_{50} values obtained for compounds in this assay are very similar to the K_i values (Table 5). The K_i values for the compounds ranged from high nanomolar to high micromolar concentrations (Table 5). Compound **18** displayed the highest affinity for mAChRs of the compounds. The Hill coefficients of all the compounds were close to unity (Table 5).

Discussion

The DMCAE homologs described in this study are pharmacologically intriguing in many respects. Several of the compounds display high-affinity binding and high potencies as nicotinic agonists and have retained the selectivity for nAChRs over mAChRs observed for MCC and DMCC. Because tertiary amines are much more likely than quaternary amines to penetrate physiological membranes, such as the blood-brain barrier, the compounds in this study are advantageous from a bioavailability perspective compared with the parent compounds MCC and DMCC.

The interaction between the quaternized amine group of ACh and the nAChR is perhaps the most meticulously studied example of all biological cation-π interactions (Zacharias and Dougherty, 2002). The quaternized amine group of ACh has been proposed to bind in a pocket constituted by five conserved aromatic residues in the nAChR (Zhong et al., 1998; Beene et al., 2002), a binding mode confirmed by the ACh-binding protein crystal structure (Brejc et al., 2001). It is noteworthy that nAChR agonists include compounds with quaternized, tertiary, and secondary amino groups as exemplified by ACh, nicotine, and epibatidine, respectively. It should be kept in mind, however, that the secondary as well as the tertiary amino groups of these compounds is almost completely but reversibly protonated at physiological pH. In a recent study, the tertiary amine analog of ACh, norACh, displayed 20-fold lower potency than ACh at the muscle-type nAChR, whereas the quaternized analog of (*S*)-nicotine, (*S*)-menicotinium, was found to be equipo-

TABLE 3

Binding characteristics of compounds to native $\alpha7$ sites and a $\alpha7/5HT_3$ chimera

The [^{3}H]MLA binding experiments with rat brain membranes and tsA cells transiently transfected with $\alpha7/5HT_3$ were performed as described under *Materials and Methods*.

Compound	K_i ($pK_i \pm$ S.E.M.)	
	Rat Brain	$\alpha7/5HT_3$
	μM	
[^{3}H]MLA		
K_D (nM)	*1.2 ± 0.3*	*1.6 ± 0.5*
B_{max} (pmol/mg of protein)	*0.32 ± 0.36*	*10.9 ± 0.86*
MLA	0.0039 (8.41 ± 0.12)	0.0019 (8.72 ± 0.02)
ACh	180 (3.74 ± 0.11)	170 (3.77 ± 0.08)
(*S*)-Nicotine	15 (4.84 ± 0.09)	31 (4.51 ± 0.07)
(−)-Cytisine	15 (4.84 ± 0.12)	48 (4.32 ± 0.09)
(±)-Epibatidine	0.24 (6.63 ± 0.08)	0.43 (6.37 ± 0.05)
Lobeline	41 (4.38 ± 0.04)	56 (7.25 ± 0.08)
MCC	44 (4.36 ± 0.07)	110 (3.96 ± 0.04)
7	N.D.	>1000 (< 3)
13	N.D.	>1000 (< 3)
14	N.D.	>1000 (< 3)
15	N.D.	>1000 (< 3)
16	N.D.	>1000 (< 3)
17	N.D.	>1000 (< 3)
18	N.D.	310 (3.51 ± 0.07)
19	N.D.	>1000 (< 3)
20	N.D.	>1000 (< 3)
21	N.D.	>1000 (< 3)

N.D., not determined.

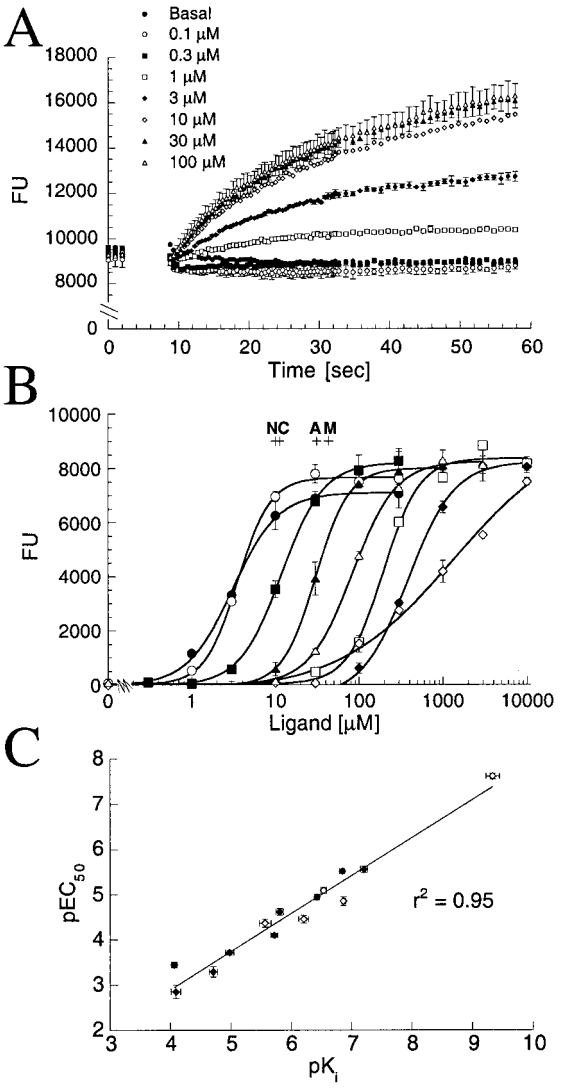

Fig. 5. Functional characterization of ligands at $\alpha3\beta4$ nAChR in a FLIPR membrane potential assay. The cells were loaded with the membrane potential-sensitive dye for 30 min and assayed in a NOVOstar plate reader as described in *Materials and Methods*. A, agonist-induced changes in the membrane potential in $\alpha3\beta4$-HEK293 cells by 8 different concentrations of compound **14**. Each data point represents the mean value of two individual wells from a 96-well plate. For reasons of clarity error bars are only depicted for the curves of concentrations 0.1, 1, 3, and 100 μM. B, concentration-response curves for compounds **7** (■), **13** (□), **14** (●), **15** (○), **17** (♦), **18** (◇), **20** (▲), and **21**(△) at $\alpha3\beta4$. For comparison, the EC_{50} values of ACh (A), (*S*)-nicotine (N), (−)-cytisine (C), and MCC (M) obtained in the individual experiment are depicted above the concentration-response curves. Data are from an individual experiment and were fitted as described under *Materials and Methods*. C, correlation between binding affinities and functional potencies of ACh, (*S*)-nicotine, (−)-cytisine, (±)-epibatidine, MCC, and compounds **7**, **13**, **14**, **15**, **17**, **18**, **19**, **20**, and **21** at the $\alpha3\beta4$-HEK293. ◇, standard ligands; ♦, carbamoylcholine homologs.

TABLE 4

Functional characteristics of compounds at the $\alpha3\beta4$ nAChR

The EC_{50} and n_H values obtained for the compounds at the $\alpha3\beta4$-HEK 293 cell line in the FLIPR membrane potential assay. The experiments were performed as described under *Materials and Methods*. Each experiment was performed in duplicate at least three times.

Compound	EC_{50} (pEC_{50} ± S.E.M.)	n_H (CI)
	μM	
ACh	35 (4.45 ± 0.09)	1.7 (1.4–2.0)
(*S*)-Nicotine	8.1 (5.09 ± 0.07)	1.8 (1.6–2.0)
(−)-Cytisine	14 (4.85 ± 0.10)	1.5 (1.3–1.7)
(±)-Epibatidine	0.024 (7.62 ± 0.06)	1.7 (1.5–1.9)
MCC	44 (4.36 ± 0.09)	1.7 (1.6–1.9)
7	11 (4.94 ± 0.06)	1.6 (1.4–1.8)
13	200 (3.71 ± 0.05)	2.1 (1.8–2.5)
14	2.8 (5.56 ± 0.07)	1.5 (1.3–1.8)
15	3.0 (5.52 ± 0.04)	2.2 (1.9–2.5)
16	N.D.	N.D.
17	360 (3.44 ± 0.06)	1.8 (1.6–2.1)
18	1,400 (2.85 ± 0.15)	0.72 (0.64–0.82)
19	520 (3.29 ± 0.12)	2.6 (2.2–3.1)
20	25 (4.61 ± 0.07)	2.1 (1.8–2.4)
21	82 (4.09 ± 0.04)	1.7 (1.4–2.1)

CI, confidence interval; N.D., not determined.

tent with (*S*)-nicotine (Beene et al., 2002). In analogy with the observations for ACh, the amino group of DMCC has to be quaternized to bind with high affinity to native nAChRs (compare DMCC and DMCAE in Table 1) (Søkilde et al., 1996). Although one has to be very cautious when comparing binding data on native neuronal nAChRs with functional data on a recombinant muscle-type nAChR, compound **7** seems to supplement ACh and (*S*)-nicotine with regard to the importance of the nature of the charge of the amino group. Whereas the potency of nicotine was unaffected by the conversion from a tertiary to a quaternized amino group, and ACh and DMCC had to possess a quaternized amino group for optimal receptor interaction, the tertiary nature of the amine group of **7** seems to be crucial for effective binding to nAChRs (compare compounds **7** and **10** in Table 1) (Søkilde et al., 1996; Beene et al., 2002). This observation indicates that the protonated amino group of **7** must bind to the "aromatic pocket" of the nAChR in a mode different from those of both the quaternized amines ACh, MCC, and DMCC and from another protonated tertiary amine, (*S*)-nicotine.

As was expected from the remarkable nicotinic selectivity of **7** displayed in binding assays to native nAChRs and mAChRs, the majority of compounds **13** to **21** were highly selective for nAChRs compared with mAChRs (Tables 1 and 5). The weak binding affinities displayed by the compounds at the $\alpha7/5\text{-}HT_3$ chimera was not surprising either, considering that $\alpha7$ is a low-affinity binding site for ACh and prototypic nicotinic agonists (Table 3). The affinities of the compounds at the $\alpha2\beta2$, $\alpha4\beta2$, $\alpha2\beta4$, $\alpha3\beta4$, and $\alpha4\beta4$ nAChRs, on the other hand, varied from low nanomolar to midmicromolar concentrations (Table 2). Furthermore, the selectivity profiles of the 10 compounds at the five α/β nAChRs were significantly different (Fig. 6). Together with the course pharmacological characterization of compounds **1** to **12** in a (*S*)-[^{3}H]nicotine binding assay labeling predominantly native $\alpha4\beta2$ nAChRs, the data constitute a detailed structure-activity study, as summarized in Fig. 7.

As mentioned above, the tertiary amine group of the DMCAE homolog was found to be crucial for its nicotinic activity. Furthermore, the optimal number of carbon atoms between the amino and carbamate groups of the molecule seemed to be three, because further elongation of the carbon backbone completely abolished binding to the nAChRs (compare compounds **1-3** in Table 1). Similarly, introduction of methyl groups in the C-1 and C-2 position of compound **1** had detrimental effects on binding affinity (compounds **8** and **9** in Table 1). This suggests that there is little space at the nAChR binding pocket for substituents in these positions of the DMCAE homologs. In contrast, the presence of a methyl group in the C-3 position led to a 70-fold increase in binding affinity for native nAChRs (compounds **1** and **7** in Table 1). The impact of the methyl group on nicotinic binding may arise from additional interactions with the nAChR or from an altered overall conformation of the molecule. In compound **21**, the space surrounding C-3 was probed further with the introduction of an ethyl group as the substituent. This compound displayed 2- to 8-fold lower affinities than **7** at the heteromeric nAChRs, but compounds **7** and **21** displayed similar binding selectivity profiles at the five α/β combinations (Table 2 and Fig. 6A). Hence, although a methyl group seemed to be optimal in the C-3 position, both methyl and ethyl groups seemed to fit into the nAChR binding pocket. Additional analogs are needed to fully explore the space surrounding the C-3 carbon and elucidate the role of this substituent in nAChR binding.

Using compounds **13** to **20**, the importance of the carbamate nitrogen substituents was investigated. Compounds **7**, **14,** and **15** with the methyl/methyl, methyl/ethyl, and ethyl/ethyl substituent combinations all displayed nanomolar binding affinities for all five α/β nAChRs. The selectivity profile of **7** was comparable with those of ACh, (*S*)-nicotine, (−)-cytisine, and MCC (Fig. 6). The affinity differences between nAChR subtypes were much smaller for compounds **14**

TABLE 5

Binding characteristics of compounds to native mAChRs

The [^{3}H]NMS competition binding assay to rat brain membranes were performed as described under *Materials and Methods*. The ratios between the binding affinities from the [^{3}H]NMS assay (K_i^{mAChR}) and the K_i values from [^{3}H]Epibatidine binding to $\alpha4\beta2$ and $\alpha3\beta4$ nAChRs are also given.

Compound	K_i (pK_i ± S.E.M)	n_H (CI)	$K_i^{mAChR}/K_i^{\alpha4\beta2}$	$K_i^{mAChR}/K_i^{\alpha3\beta4}$
	μM			
7	51 (4.29 ± 0.08)	0.85 (0.81–0.89)	230	140
13	74 (4.13 ± 0.02)	0.96 (0.73–1.3)	5700	7.1
14	9.7 (5.01 ± 0.05)	0.81 (0.75–0.89)	1700	160
15	6.6 (5.18 ± 0.01)	0.88 (0.81–0.95)	550	47
17	2.9 (5.53 ± 0.05)	0.88 (0.84–0.92)	1.9	0.034
18	0.55 (6.26 ± 0.05)	0.96 (0.78–1.2)	0.011	0.0068
19	4.2 (5.38 ± 0.02)	0.97 (0.84–1.1)	33	0.21
20	4.1 (5.38 ± 0.09)	0.82 (0.72–0.92)	33	2.7
21	48 (4.32 ± 0.03)	0.98 (0.85–1.1)	340	25

CI, confidence interval.

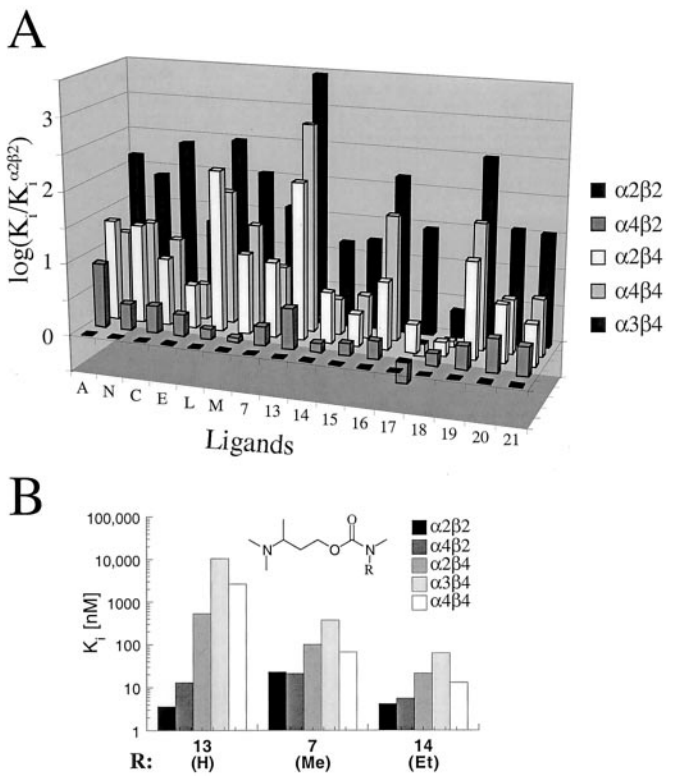

Fig. 6. Binding selectivity profiles of carbamoylcholine homologs at heteromeric nAChRs. A, selectivity profiles of standard nicotinic ligands and compounds **7** and **13** to **21** at $\alpha2\beta2$, $\alpha4\beta2$, $\alpha2\beta4$, $\alpha3\beta4$, and $\alpha4\beta4$ nAChRs. ACh, (*S*)-nicotine, (−)-cytisine, (±)-epibatidine, lobeline, and MCC are depicted as A, N, C, E, L, and M, respectively. B, role of carbamoyl substituent (R) for the selectivity profiles of compounds **7**, **13** and **14**. The respective carbamoyl substituents (R) are given below the compound numbers.

and **15**, the difference between the K_i values of **14** for $\alpha4\beta2$ and $\alpha3\beta4$ being only 11-fold (Fig. 6B). Strikingly, compound **13,** with its methyl/hydrogen substituent combination, displayed a completely opposite selectivity profile (Table 2 and Fig. 6B). Whereas the compound exhibited low nanomolar affinities for $\alpha2\beta2$ and $\alpha4\beta2$, it was more than 100-fold weaker at the corresponding $\beta4$-containing nAChRs, $\alpha2\beta4$, and $\alpha4\beta4$. The 800-fold weaker binding affinity for $\alpha3\beta4$ than for $\alpha4\beta2$ displayed by this compound is comparable in magnitude to the reported $K_i^{\alpha3\beta4}/K_i^{\alpha4\beta2}$ ratio of A-85380 (Mukhin et al., 2000). It is well documented that $\beta2$-containing nAChRs in general display higher binding affinities for standard nicotinic agonists than $\beta4$-containing nAChRs (Parker et al., 1998), and the differences have been proposed to arise primarily from the interactions of the agonists with the so-called loop E of the β-subunit (Cohen et al., 1995). Based on a molecular model of the N-terminal region of the $\alpha4\beta2$ pentamer, the Phe^{116} residue of $\beta2$ and the corresponding Gln^{117} in $\beta4$ have been suggested as the principle residues responsible for this difference (Le Novère et al., 2002). Although it is tempting to speculate on the role of loop E in the binding of compounds **7**, **13,** and **14**, detailed structural studies are needed to fully elucidate these interactions.

In contrast to the high nAChR binding affinities observed with small aliphatic groups as carbamate *N*-substituents, introduction of bulkier groups led to dramatically decreased binding affinities (Table 2). Whereas replacement of one of the methyl groups of **7** with a propyl group caused only a moderate decrease in binding affinity, the presence of two propyl groups dramatically decreased nAChR binding affinity (compounds **16** and **17**). Introduction of two phenyl groups at the carbamate nitrogen abolished nicotinic activity almost completely; compound **18** was a selective mAChR rather than nAChR ligand (Tables 2 and 5). Compounds **19** and **20**, in which the carbamate nitrogen had been replaced with a pyrrolidine and a piperidine ring, respectively, also displayed reduced binding affinities compared with **7**, although the impairment was less pronounced (Table 2).

Compounds **7** and **13** to **21** were agonists at the $\alpha3\beta4$ nAChR (Fig. 5B and Table 4). The compounds are likely to be agonists at the other nAChR subtypes as well, although this must be confirmed in future studies. Considering the therapeutic prospects of a nicotinic agonist capable of discriminating between the $\alpha4\beta2$ and the ganglionic $\alpha3\beta4$ subtype (Holladay et al., 1997; Lindstrom, 1997; Arneric and Brioni, 1999), the 800-fold difference in binding affinities of compound **13** between these two subtypes is noteworthy (Table 2 and Fig. 6B). However,

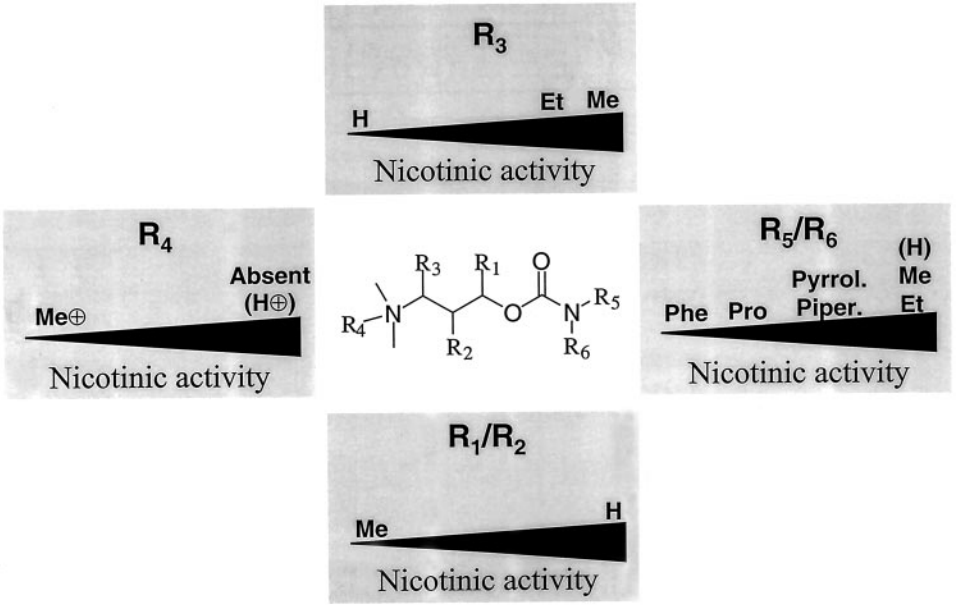

Fig. 7. Structure-activity relations for the DMCAE homologs at nAChRs. Et, ethyl; H, hydrogen; Me, methyl; Phe, phenyl; Piper., piperidine; Pro, propyl; Pyrrol., pyrrolidine.

because equilibrium binding and functional characterization reflect the affinity of the agonist for the desensitized and activated state of the nAChR, respectively, a clear-cut correlation between affinity and potency cannot be assumed. In agreement with this, very different findings on the correlation between binding and functional data have been reported from studies on epibatidine and cytisine analogs at various recombinant neuronal nAChRs (Avalos et al., 2002; Slater et al., 2003). In this study, we observed a clear correlation between binding and functional properties of the compounds at the $\alpha3\beta4$ nAChR (Fig. 5C). It remains to be seen, however, whether the binding selectivity profiles of the compounds at other α/β nAChRs also are mirrored by their functional properties and whether compound **13** also is a $\beta2$-selective nAChR agonist in functional assays.

In conclusion, this series of DMCAE homologs includes the most potent and selective nicotinic agonists yet derived from ACh and CCh. The fact that these compounds are tertiary amines is interesting from a bioavailability perspective and suggests a binding mode to the nAChRs different from those of ACh and (*S*)-nicotine. Compounds **7** to **21** in this study are racemic mixtures, and the enantiomers will have to be resolved and characterized pharmacologically in future studies. Considering the highly flexible nature of the DMCAE homolog molecule, it may be possible to increase binding affinity and potency even further by introduction of conformational restraints in the molecule. Finally, the pronounced selectivity for $\beta2$-containing nAChRs observed for compound **13** in binding assays is intriguing, and compounds **14** and **15** could be potential leads for series of $\beta4$-preferring ligands. All in all, the 3-*N*,*N*-dimethylaminobutylcarbamate structure looks promising for the future development of subtype selective nicotinic agonists.

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