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Untranslated Region-Dependent Exclusive Expression of High-Sensitivity Subforms of $\alpha4\beta2$ and $\alpha3\beta2$ Nicotinic Acetylcholine Receptors[S]

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ABSTRACT

$\alpha4\beta2$ nicotinic acetylcholine receptors (nAChRs) are recognized as the principal nicotine binding site in brain. Recombinant $\alpha4\beta2$ nAChR demonstrate biphasic concentration-response relationships with low- and high-EC_{50} components. This study shows that untranslated regions (UTR) can influence expression of high-sensitivity subforms of $\alpha4\beta2$ and $\alpha3\beta2$ nAChR. Oocytes injected with $\alpha4$ and $\beta2$ RNA lacking UTR expressed biphasic concentration-response relationships for acetylcholine with high-sensitivity EC_{50} values of 0.5 to 2.5 μM (14–24% of the population) and low-sensitivity EC_{50} values of 110 to 180 μM (76–86%). In contrast, message with UTR expressed exclusively the high-sensitivity $\alpha4\beta2$ nAChR subform with an acetylcholine EC_{50} value of 2.2 μM. Additional studies revealed pharmacological differences between high- and low-sensitivity $\alpha4\beta2$ subforms. Whereas the antagonists dihydro-β-erythroidine (IC_{50} of 3–6 nM) and methyllycaconitine (IC_{50} of 40–135 nM) were not selective between high- and low-sensitivity $\alpha4\beta2$, chlorisondamine, mecamylamine, and *d*-tubocurarine were, respectively, 100-, 8-, and 5-fold selective for the $\alpha4\beta2$ subform with low sensitivity to acetylcholine. Conversely, agonists that selectively activated the high-sensitivity $\alpha4\beta2$ subform with respect to efficacy as well as potency were identified. Furthermore, two of these agonists were shown to activate mouse brain $\alpha4\beta2$ as well as the ferret high-sensitivity $\alpha4\beta2$ expressed in *Xenopus laevis* oocytes. With the use of UTR-containing RNA, exclusive expression of a novel high-sensitivity $\alpha3\beta2$ nAChR was also achieved. These studies 1) provide further evidence for the existence of multiple subforms of $\alpha4\beta2$ nAChR, 2) extend that to $\alpha3\beta2$ nAChR, 3) demonstrate UTR influence on $\beta2$-containing nAChR properties, and 4) reveal compounds that interact with $\alpha4\beta2$ in a subform-selective manner.

Nicotinic acetylcholine receptors (nAChRs) are a diverse group of ligand-gated ion channels found in brain and spinal cord; autonomic, enteric, and sensory nervous systems; skeletal muscle; cochlea; and several non-neuronal cell types (Alkondon and Albuquerque, 2004; Champtiaux and Changeux, 2004; Gotti and Clementi, 2004; Hogg and Bertrand, 2004). These receptors are defining members of the pentameric superfamily, including 5-hydroxytryptamine$_3$, $GABA_A$, and glycine receptors. Functional receptors are comprised by at least one "α" subunit, which contains signature sequences required for binding and channel activation. However, most nAChR also require non-α subunits to form a functional complex, which together with the pentameric structure could permit formation of multiple functionally distinct nAChR from even just two different subunits [e.g., $\alpha4_{(2)}\beta2_{(3)}$ and $\alpha4_{(3)}\beta2_{(2)}$] (Zhou et al., 2003). In mammalian brain, nine subunits predominate—$\alpha2$ through $\alpha7$ and $\beta2$ through $\beta4$—and, among these, only $\alpha7$ can form homomeric functional pentamers (Champtiaux and Changeux, 2004; Gotti and Clementi, 2004).

Despite the potential huge diversity of nAChR, most CNS functions have been ascribed to $\alpha4\beta2$, $\alpha3$-containing ($\alpha3$*), $\alpha6$*, and $\alpha7$ nAChR. In particular, approximately 90% of the high-affinity nicotine binding sites in rat brain consist of $\alpha4\beta2$ (Clarke et al., 1985; Whiting et al., 1987; Flores et al.,

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; CNS, central nervous system; UTR, untranslated region; DHβE, dihydro-β-erythroidine; PCR, polymerase chain reaction; A-163554, (*R*)-2-chloro-3-(5,5-dimethyl-hexa-1,3-dienyl)-5-(pyrrolidin-2ylmethoxy)pyridine dihydrochloride; A-162035, (*R*)-2-chloro-3-phenyl-5-(pyrrolidin-2-ylmethoxy)-pyridine hydrochloride; A-168939, (*R*)-5-chloro-6-(2-pyridin-4-yl-vinyl)-2-pyrrolidin-2-yl-furo[3,2-*b*]pyridine dihydrochloride; A-84543, 3-[2-((*S*)-pyrrolidinyl)methoxypyridine; TM, transmembrane; ORF, open reading frame; CI, confidence interval; MLA, methyllycaconitine.

1992; Zoli et al., 1995; Champtiaux et al., 2003). From a functional perspective, native $\alpha4\beta2$ nAChR EC_{50} values for nicotine and the neurotransmitter acetylcholine are in the low micromolar range (Alkondon and Albuquerque, 1993; Marks et al., 1993, 1999), 1 to 2 orders of magnitude lower than for other nAChR and consistent with higher affinity binding to $\alpha4\beta2$. In contrast, recombinant $\alpha4\beta2$ nAChR expressed in oocytes and mammalian cell lines have demonstrated variable functional potencies for acetylcholine and nicotine with lower sensitivity (>40 μM) EC_{50} values (Gopalakrishnan et al., 1996; Chavez-Noriega et al., 1997; Sabey et al., 1999; Papke et al., 2000) as well as the higher sensitivity ($\leq$3 μM) EC_{50} values (Court et al., 1994; Papke and Heinemann, 1994; Buisson et al., 1996; Gopalakrishnan et al., 1996; Kuryatov et al., 1997; Olale et al., 1997; Labarca et al., 2001). Indeed, individual cells may express both high- and low-sensitivity forms of recombinant $\alpha4\beta2$ and $\alpha4\beta4$ (Covernton and Connolly, 2000; Houlihan et al., 2001) in a proportion that may be influenced by $\alpha4$ polymorphism (Kim et al., 2003), by $\beta2$ content (Zwart and Vijverberg, 1998; Buisson and Bertrand, 2001; Nelson et al., 2003; Zhou et al., 2003), or by prolonged (overnight) exposure to nicotine or low temperature (Buisson and Bertrand, 2001; Nelson et al., 2003). However, it is not clear whether low- as well as high-sensitivity $\alpha4\beta2$ nAChR are expressed in CNS, what their respective roles in behavior or development may be, or how the proportion of high- and low-sensitivity forms may be regulated apart from long-term exposure to nicotine.

In this study, we present evidence that untranslated regions (UTRs) of the nAChR transcripts influence the expression of high- and low-sensitivity nAChR to the extent of permitting exclusive expression of the high-sensitivity $\alpha4\beta2$ nAChR subform. This property does not seem to be limited to $\alpha4\beta2$ nAChR but extends at least to $\alpha3\beta2$ nAChR as well.

Among the various nAChR, $\alpha4\beta2$ are unusual in that they are potentiated rather than inhibited by the neuroactive steroid 17β-estradiol (Nakazawa and Ohno, 2001; Curtis et al., 2002) through a mechanism involving the carboxyl terminus of the $\alpha4$ subunit (Paradiso et al., 2001). Estradiol also potentiated ferret $\alpha4\beta2$ nAChR, and with apparently greater effect on the high-sensitivity subform. Thus, $\alpha4\beta2$ physiology may be regulated through selective modulation by endogenous substances as well as through expression of nAChR with differing sensitivity to the neurotransmitter acetylcholine.

We also evaluated the selectivity of several antagonists and agonists to identify compounds that may be useful for examining the roles of high- and low-sensitivity $\alpha4\beta2$ nAChR. With both high-sensitivity and mixed sensitivity forms of $\alpha4\beta2$, dihydro-β-erythroidine (DHβE) and methyllycaconitine were potent antagonists but did not seem to distinguish between the high- and low-sensitivity subforms. In contrast, mecamylamine, *d*-tubocurarine, and chlorisondamine were 8-, 5-, and 100-fold selective for the low-sensitivity form. None of the antagonists examined was selective for the high-sensitivity $\alpha4\beta2$, which, is the form more sensitive to acetylcholine. In contrast, some agonists did seem to be very selective for the high-sensitivity $\alpha4\beta2$ nAChR subform. Two of these agonists were shown to be active at mouse brain $\alpha4\beta2$ nAChR as well as at ferret high-sensitivity $\alpha4\beta2$ nAChR expressed in oocytes, supporting the idea that the high-sensitivity $\alpha4\beta2$ nAChR subform is expressed in brain.

Materials and Methods

Total RNA was prepared from ferret brain (Analytical Biological Services, Inc., Wilmington, DE) with the use of TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Poly-A+ RNA was isolated with the use of the Oligotex mRNA system (QIAGEN, Valencia, CA). Two different methods were used in cloning the nAChR subunits. One method involved identification of a full-length clone from a cDNA library, whereas the other method used standard PCR techniques to amplify fragments. The cDNA library screening used for $\alpha4$ and $\beta2$ provided coding sequence and genomic 5′- and 3′-UTRs, whereas the PCR method used for $\alpha3$, $\alpha4$, and $\beta2$ generated coding sequence without the UTR.

Isolation of $\alpha4$ and $\beta2$ from a cDNA Library. cDNA was synthesized from ferret brain poly-A+ RNA by using the Orient Express kit with random primers, ligated to EcoRI/HindIII linkers (Novagen, Madison, WI), and digested with EcoRI + HindIII (New England Biolabs, Beverly, MA). The cDNA was fractionated on a sucrose gradient to remove material smaller than 500 bp. The vector pcDNA3.1(−) (Invitrogen) was digested with EcoRI and HindIII, treated with calf intestinal alkaline phosphatase, and purified over a Chromaspin-TE 1000 column (Clontech, Mountain View, CA). Vector and cDNA were ligated with a Novagen DNA ligation kit and transformed into ElectroMax DH10B cells (Invitrogen) by electroporation. The electroporation mixture was diluted to approximately 1000 transformants/ml in autoclaved 2% tryptone, 1% yeast extract, 1% NaCl, 0.3% SeaPrep agarose (Cambrex Bio Science Rockland, Inc., Rockland ME); equilibrated to 37°C; and supplemented with 100 mg/ml ampicillin. Aliquots (40 ml) were poured into sterile 50-ml tubes, chilled in iced water for 30 min to solidify the agarose, and incubated at 30°C for 2 days. Tubes were inverted several times to mix colonies, and a small aliquot from each tube was stored at −80°C in 15% glycerol. The remaining cells were centrifuged, and plasmid DNA isolated with REAL Prep 96 kit (QIAGEN). In total, 384 library aliquots (four 96-well plates) were prepared, representing approximately 20 million clones.

For library screening, plasmid DNA was denatured with base and spotted on positively charged nylon membranes (Roche Molecular Biochemicals, Indianapolis, IN) with a 96-pin device (V&P Scientific, San Diego, CA). The membranes were neutralized, and the DNA was fixed by UV exposure (Stratalinker; Statagene, La Jolla, CA). Membrane replicates were then hybridized individually to various oligonucleotides that had been labeled with T4 polynucleotide kinase (Invitrogen) and [γ-^{32}P]ATP, washed at varying stringencies, and exposed at −80°C with Kodak BioMax intensifying screens and BioMax film. The following oligonucleotide probes were prepared according to a design based upon homology to published nAChR subunits and to partial ferret sequence data derived from short PCR fragments: oligonucleotide 1, GCCGCTCTTCTACACCATCAACCTCATC (highly conserved for all α and β nAChR subunits); oligonucleotide 2, GAACGGTTGCTGAAGACACTCTTCTCCGGCTACAACAAGTGGTC (ferret $\alpha4$, N-terminal half); oligonucleotide 3, GGCGGCTCATCGAGTCCATGCACAAGGTGGCCAGCGCCCC (ferret $\alpha4$, C-terminal half); oligonucleotide 4, GAGCGGCTAGTGGAGCATCTCCTGGACCCCTCCCGGTACAACAAG (ferret $\beta2$, N-terminal half); and oligonucleotide 5, ACCATCGGCATGTTCCTGCAGCCTCTCTTCCAGAACTACAC (human $\beta2$, C-terminal half).

Based upon hybridization signals, individual library aliquots believed to contain full-length $\alpha4$ and $\beta2$ subunit cDNA clones were identified. Colonies from each were plated onto agar, grown, transferred to nylon membranes, and screened with oligonucleotide probes. For $\alpha4$, a mixture of oligonucleotides 1, 2, and 3 was used; for $\beta2$, a mixture of oligonucleotides 1, 4, and 5 was used. Individual colonies were identified and characterized. All $\alpha4$ colonies were found to contain identical inserts for the complete coding sequences plus 5′ and 3′ noncoding regions. There were two different cDNA inserts for $\beta2$; one insert began with 5′ noncoding sequences and extended toward the middle, whereas the other insert began in the middle coding region and ended in 3′ noncoding sequences. Because there were several hundred nucleotides

of overlap between the latter two clones that included a unique BsgI restriction site, a series of restriction digestions and ligations were used to produce a full-length $\beta2$ clone.

Isolation of $\alpha3$, $\alpha4$, and $\beta2$ cDNAs by PCR. cDNA prepared from either total RNA or poly-A+ RNA was amplified by PCR with the use of the Superscript II preamplification system (Invitrogen) and either oligo(dT) or random hexamer primers. First strand cDNA synthesis was performed according to the manufacturer's instructions. In brief, the RNA was primed with either random hexamers or oligo(dT) in the presence of dNTPs, and reactions were initiated by the addition of 50 U of Superscript II reverse transcriptase. After termination of the reaction, the remaining RNA template was removed by treatment with 2 U of RNase H, and partial cDNAs were then amplified by PCR with the use of gene-specific primers. Primers were designed to correspond to areas that are divergent from sequences of other nAChR subunits but show relatively good homology between human and rat cDNA sequences of the desired nAChR subunit. In some instances, degenerate primers were used. DNA sequences were amplified by PCR with the use of either Advantage HF, Advantage HF2 (Clontech), or Amplitaq Gold (PerkinElmer Life and Analytical Sciences, Boston, MA) polymerases. In brief, for $\beta2$, after initial template denaturation for 3 min at 94°C, amplification was performed with thermal cycles of 94°C for 30 s, followed by 68°C for 3 min for 35 cycles (two-step PCR), followed by a final extension at 68°C for 7 min. For $\alpha4$ and $\alpha3$, the template was denatured for 30 s at 94°C, and amplification was performed with thermal cycles of 94°C for 15 s, followed by 68°C for 3 min for 35 cycles, followed by a final extension at 68°C for 7 min. In some instances, PCR was performed in the presence of 5% dimethyl sulfoxide or by using Advantage-GC2 (Clontech), when specific GC-rich areas of the cDNAs were unobtainable under more standard PCR conditions. A 20-μl aliquot of the reaction was run on a 1% agarose gel and PCR products of the expected size were extracted with the use of the QIAquick kit (QIAGEN), cloned into the pCR 2.1-TOPO vector (Invitrogen), and expanded with the use of One Shot TOP 10 chemically competent *Escherichia coli* (Invitrogen) in preparation for sequencing. DNA sequences were identified and confirmed with overlapping sequences generated from different PCR primer sets. For the $\alpha3$ nAChR subunit, four overlapping partial cDNA clones were used to construct a full-length clone, for the $\alpha4$ subunit three overlapping clones were used, and for the $\beta2$ cDNA four overlapping clones were used. Primer sequences used to generate these partial cDNAs are shown in Table 1.

Full-length cDNA was prepared by using gene splicing by overlap extension and PCR amplification; resultant cDNA was confirmed by sequencing. These clones contained minimal or no 5′- or 3′-UTR sequence. cDNAs were subcloned into mammalian expression vectors by EcoRI digestion of the plasmids in the pCR2.1-TOPO vector, cDNA fragment isolation from 1% agarose gels, and subsequent ligation into the EcoRI site of either pcDNA3.1(−)Hygro (for the $\alpha4$ and $\alpha3$ subunits) or pcDNA3.1(−) (for the $\beta2$ subunit). Capped cRNA was prepared by using mMessage mMachine (Ambion, Austin, TX) transcription via the T7 promoter in the pcDNA 3.1 vector.

Expression of nAChR in *Xenopus laevis* Oocytes. Female *X. laevis* frogs were obtained from Nasco (Fort Atkinson, WI) and were maintained and treated with standard protocols approved by Abbott's Institutional Animal Care and Use Committee. The preparation of *X. laevis* oocytes, injection with cDNA or cRNA prepared by standard techniques, and measurement of nAChR responses by using two-electrode voltage-clamp followed procedures similar to those described previously (Briggs et al., 1995). In brief, ovaries were removed surgically from a *X. laevis* frog under tricaine anesthesia (0.28% in deionized water), and oocytes were prepared after incubation for 1 to 2 h at room temperature in 2 mg/ml collagenase (Sigma type 1A) in low-Ca^{2+} Barth's solution, pH 7.55, containing 87.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM Na-HEPES, and 100 μg/ml gentamicin. Oocytes were maintained, before and after injection, at

TABLE 1
Primers

$\alpha3$ nAChR	
Set 1	
Sense	5′-CTCCAGGTCTGGGGTCTGCGCTG-3′
Antisense	5′-GCTTTGGTCTTGTCGTCCACCTGG-3′
Set 2	
Sense	5′-GCCAGTGGCCAGGGCCTCAGAGGC-3′
Antisense	5′-CCCAGTAGTCCTTGAGGTTCATGG-3′
Set 3	
Sense	5′-CCATGAACCTCAAGGACT-3′
Antisense	5′-CACCATGGCAACATATTCC-3′
Set 4	
Sense	5′-GCCAAAGAGATTCAAGATGATTGGAAGTATGTTGCCATGG-3′
Antisense	5′-TCTATGTGTCATCTCTGGCCATCAAGGGTTGCAG-3′
$\alpha4$ nAChR	
Set 1	
Sense	5′-TGCGTGCGCCATGGAGCTAGGGGGC-3′
Antisense	5′-CGTACGTCCAGGAGCCGAACTTCATG-3′
Set 2	
Sense	5′-ACGGRMGGGTGCAGTGGA-3′
Antisense	5′-CTTCTGGCCMGAGCCWG-3′
Set 3	
Sense	5′-CGGCCCTCCGTGGTCAAGGACAACT-3′
Antisense	5′-TCCTAGATCATRCCAGCCA-3′
$\beta2$ nAChR	
Set 1	
Sense	5′-CGGCTTCAGCACCACGGACAGCGCCCCACC-3′
Antisense	5′-CCGAGACTCGACCACTGACATGTCGAGTACC-3′
Set 2	
Sense	5′-ACKGAYACAGAGGAGCGG-3′
Antisense	5′-GAAGATAAGGTTACGRCACC-3′
Set 3	
Sense	5′-TCACMTGGAAGCCTGARGA-3′
Antisense	5′-GGTAGCAGTGGTCGCACA-3′
Set 4	
Sense	5′-GCGGCGAGAAGATGACGCTGTGCATCTCCG-3′
Antisense	5′-GGTAGCAGTGGTCGCACA-3′

17–18°C in normal Barth's solution, pH 7.55, containing 90 mM NaCl, 1 mM KCl, 0.66 mM $NaNO_3$, 0.74 mM $CaCl_2$, 0.82 mM $MgCl_2$, 2.4 mM $NaHCO_3$, 2.5 mM sodium pyruvate, 10 mM Na-HEPES buffer, and 100 μg/ml gentamicin. Glass Petri dishes were used to avoid any potential interference with nAChR function by substances found in some plastics (Papke et al., 1994).

For expression of nAChR, oocytes were injected within 24 h of their preparation and were used 2 to 7 days after injection. Each oocyte was injected with either 40 to 50 nl of nAChR RNA or 10 to 15 nl of nAChR DNA. The total concentration of RNA or DNA was approximately 1 μg/μl determined spectrophotometrically. Injections were conducted with the use of like preparations only (e.g., RNA with RNA or DNA with DNA). Results were similar with either RNA or DNA, but RNA was preferred in studies with varied message ratios to avoid transcription variance.

For measuring functional nAChR responses, oocytes were transferred to room temperature OR-2 solution, pH 7.4, containing 90 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM Na-HEPES buffer, and 0.5 μM atropine to block endogenous muscarinic receptors. In some experiments, $CaCl_2$ was replaced by $BaCl_2$ to prevent secondary activation of a Ca^{2+}-dependent Cl^- current. Compounds were applied, and responses were measured at −60 mV cell potential in the POETs apparatus, a computer-controlled robotic device that controls compound delivery, electrophysiological response recording, and data storage and measurement in a searchable database (Trumbull et al., 2003). The device operates six oocyte-containing chambers, applies compounds using a robotic pipettor (Gilson, Middleton, WI) (typically, 4 ml/min for 4 s followed by 3- to 5-min wash by perfusion), records responses under two-electrode voltage clamp using Geneclamp 500 amplifiers (Molecular Devices, Sunnyvale, CA), National Instruments analog-to-digital converter (Austin, TX), and an IBM-compatible computer. Custom software was used to schedule compound application to the oocytes at user-defined intervals (typically 3–5 min), store the recordings in a searchable database, retrieve the responses, quantify the responses by peak amplitude or integral, and perform curve fitting or export the data to other software for further analysis. For the data presented here, concentration-response parameters were determined by nonlinear curve-fitting in Prism (GraphPad Software, San Diego, CA) and the built-in variable slope sigmoidal curve (Hill equation) or a biphasic version that was the sum of two independent Hill equations. In general, the concentration-response parameters for curve fitting were not constrained except that the bottom of the curve was set equal to 0; exceptions are noted.

In each oocyte, responses to test compound were normalized to the maximal response to acetylcholine (100 μM or 1 mM as indicated, depending upon the nAChR), and the stability of responses during testing was monitored by applying acetylcholine at regular intervals during the experiment. Agonist responses typically were measured as the compound-induced peak (maximal) inward current relative to the baseline holding current. In some experiments, the response integral ("area under the curve") also was measured, with the beginning and end of the integration period defined by the beginning and end of the activation of the Gilson syringe pump used to apply compound. Similar concentration-response parameters were obtained by integral or peak amplitude.

Mouse Brain Synaptosome Rubidium Flux. To assess agonist potency and efficacy at native $\alpha4\beta2$ nAChR, DHβE-sensitive stimulation of $^{86}Rb^+$ efflux from mouse thalamic synaptosomes was determined as described by Marks et al. (1999, 2004). C57BL/6J mice were bred at the Institute for Behavioral Genetics (University of Colorado, Boulder, CO) and were treated as approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder. The crude synaptosomal fraction was prepared by hand homogenization (Teflon-glass tissue grinder) in 10 volumes of ice-cold 0.32 M sucrose with 5 mM HEPES buffer, pH 7.5. The homogenate was centrifuged at 500*g* for 10 min to pellet nuclei and heavy debris (P1), and the supernatant subsequently was centrifuged at 12,000*g* for 20 min to yield the synaptosomal pellet (P2). To load the synaptosomes with $^{86}Rb^+$, the P2 was resuspended in uptake buffer (140 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, and 25 mM Na-HEPES buffer, pH 7.5) and incubated with 4 μCi of ^{86}RbCl for 30 min in a final volume of 35 μl. Uptake was terminated by filtration onto a glass fiber filter (Gelman type AE; 6 mm in diameter) and two 0.5-ml washes with uptake buffer. For experimental measurements, the loaded filter was transferred to a polypropylene platform and perfused at 2.5 ml/min with buffer containing 135 mM NaCl, 5 mM CsCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, 25 mM Na-HEPES buffer, pH 7.5, 50 nM tetrodotoxin, 1 μM atropine, and 0.1% bovine serum albumin fraction V. Compounds were applied by filling a 200-μl loop with appropriate solution and diverting perfusion buffer through the loop by means of a four-way rotary Teflon injection valve. Efflux of $^{86}Rb^+$ was detected continuously by pumping perfusate through a 200-μl flow-through Cherenkov cell in a β-RAM radioactivity high-performance liquid chromatography detector (IN/US Systems Inc., Tampa, FL).

Total agonist-stimulated responses were calculated as the increase in signal above the basal efflux rate, which was calculated by a nonlinear least-squares fit of the data before and after the peak response (Marks et al., 1999, 2004). Responses were normalized by dividing the agonist-stimulated response by the basal efflux. Each experiment also included samples stimulated with 10 μM nicotine to facilitate comparison of results between experiments.

Materials. Acetylcholine, atropine, bovine serum albumin, collagenase type IA, dihydro-β-erythroidine, 17β-estradiol, gentamicin, mecamylamine, methyllycaconitine, (−)-nicotine tartrate, and *d*-tubocurarine were purchased from Sigma Chemical Co. (St. Louis, MO). Chlorisondamine was purchased from Tocris Cookson Inc. (Ellisville, MO). HEPES and sucrose were from Boehringer-Ingelheim (Indianapolis, IN). CsCl and Budget Solve scintillation fluid were from Research Products International (Mt. Prospect, IL). Carrier-free ^{86}RbCl was from PerkinElmer Life and Analytical Sciences (Boston, MA). A-163554, A-162035, and A-168939 were synthesized at Abbott Laboratories as described by Lin et al. (2001).

Results

In cloning ferret $\alpha4$ and $\beta2$ nAChR, two approaches were used. One approach involved the use of primers designed to encompass the coding region with minimal 3′ and 5′ exten-

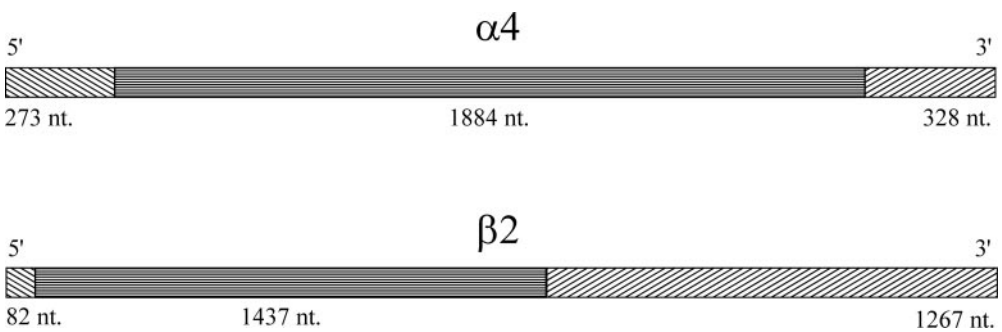

Fig. 1. Ferret $\alpha4$ and $\beta2$ UTR. The sizes of the 5′-UTR (top-down diagonal) and 3′ UTR (bottom-up diagonal) segments of ferret $\alpha4$ and $\beta2$ cloned from the cDNA library are diagrammed relative to the length of the coding sequences. The $\beta2$ 3′-UTR (1267 nucleotides) was nearly as long as the coding region (1437 nucleotides). Complete sequences are shown in Supplemental Data.

sion, and the other approach involved the use of a cDNA library with oligonucleotide probes directed toward the coding regions. Because the latter approach is based upon hybridization to long, potentially full-length cDNA derived from mRNA, it permits isolation of cDNAs containing UTR. Indeed, α4 and β2 messages with relatively long 3′- and 5′-UTR were isolated by the cDNA library screening. The relative sizes of the ferret α4 and β2 nAChR UTR segments are diagrammed in Fig. 1. The Supplemental Data shows ferret α3, α4, and β2 nAChR amino acid sequences and ferret α4 and β2 nAChR UTR nucleotide sequences aligned with corresponding human and rat sequences.

Expression of High- and Low-Sensitivity α4β2 nAChR. Oocytes injected with RNA or DNA derived from these clones expressed functional α4β2 nAChRs, but with different results depending upon whether the messages contained UTR sequences. In the following, "α4(u)" refers to α4 coding sequence with 5′- and 3′-UTR; likewise, "β2(u)" refers to β2 coding sequence with 5′- and 3′-UTR.

Oocytes injected with ferret α4 and β2 (1:1 ratio) lacking UTR expressed typical acetylcholine-gated currents and a biphasic concentration-response relationship as reported previously for human and rat α4β2 (Zwart and Vijverberg, 1998; Chavez-Noriega et al., 2000; Buisson and Bertrand, 2001; Nelson et al., 2003). In contrast, when α4(u) and β2(u) were used, the concentration-response relationship was monophasic with an EC_{50} value similar to the high-sensitivity portion of the biphasic relationship seen with the use of messages without the UTR. Concentration-response relationships for acetylcholine are shown in Fig. 2, and extracted parameters are given in Table 2. In view of the unexpected results with the α4(u)β2(u) combination, the initial measurements were repeated in 30 oocytes from three donor *X. laevis* with similar results from each cell.

Zwart and Vijverberg (1998) reported that increasing the proportion of β2 message to an α4/β2 ratio of 1:9 could lead to the appearance of a biphasic concentration-response curve with expression of a higher sensitivity component. Reasoning that the effect we observed may result from higher levels of β2 protein caused by increased translation of β2 owing to the presence of UTR, we attempted to generate monophasic high-sensitivity acetylcholine concentration curves by adjusting the α4/β2 ratio by using messages without UTR. Decreasing the α4/β2 ratio to as much as 1:120 increased the high-sensitivity proportion (Fig. 3; Table 2); however, the acetylcholine concentration-response curves remained biphasic. Thus, we were unable express exclusively monophasic high-sensitivity ferret α4β2 using messages lacking UTR.

To determine whether α4 UTR or β2 UTR was required for exclusive expression of the high-sensitivity α4β2 subform, α4 with or without UTR was combined with β2 with or without UTR. When 1:1 ratios were used, UTR in both α4 and β2 seemed to be required (Fig. 4) because the low-sensitivity subform clearly was expressed when either α4 without UTR or β2 without UTR was used. However, the β2 with UTR seemed to have the greater effect and could increase the expression of the high-sensitivity α4β2 subform even when α4 lacked UTR (Fig. 4C; Table 2). Consistent with this, in further experiments it was found that high-sensitivity α4β2 could be exclusively expressed by using α4 lacking UTR plus β2 with UTR in a ratio of 1:5 α4/β2(u) (Fig. 5).

Expression of High- and Low-Sensitivity α3β2 nAChR. The above-mentioned observations suggested that β2(u) could regulate the form of α4β2 nAChR expressed in oocytes. To determine whether this effect may generalize to other β2-containing nAChR, ferret α3 was combined with β2 and β2(u) in ratios ranging from 1:1 to 1:20 α3/β2. The acetylcholine-concentration-response curve for α3β2 1:1 could be fit with a biphasic curve and EC_{50} values of 25 and 450 μM (Fig. 6; Table 3). However, when β2(u) was used, a lower EC_{50} (3–9 μM) component occurred, predominated at an α3/β2(u) message ratio of 1:10, and was exclusively expressed at an α3/β2(u) message ratio of 1:20. Without β2-UTR, however, exclusive expression of the high-sensitivity α3β2 subform could not be achieved at a message ratio up to 1:20. Thus, β2(u) seemed to regulate expression of higher-sensitivity forms of α3β2 as well as α4β2.

Antagonist Potency at α4β2 Subforms. It remains unclear whether native α4β2 nAChR are better represented by the higher-sensitivity form, the lower sensitivity form, or whether both forms may be expressed and regulated differ-

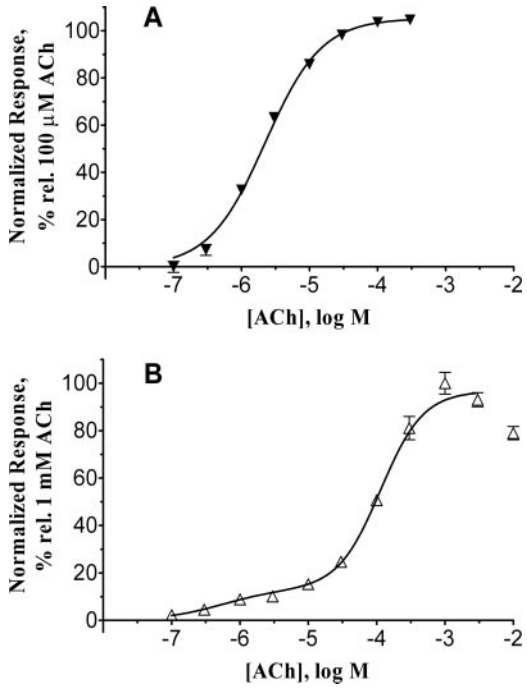

Fig. 2. Concentration-response curves for acetylcholine at ferret α4β2 nAChR expressed from messages with and without UTR. Ferret nAChR subunits were expressed in *X. laevis* oocytes by using standard techniques and responses to acetylcholine applied for 5 s were obtained at −60 mV and measured at peak amplitude relative to the baseline holding current. In each oocyte, responses to various concentrations of acetylcholine were normalized to reference control responses to account for cell to cell variance in level of receptor expression. The reference control, designed to elicit a maximal response (100 μM acetylcholine for high-sensitivity α4β2 from UTR-containing messages and 1 mM acetylcholine for other nAChR), was contained in a solution separate from the test solutions, and was applied at regular intervals during the experiment to detect fluctuations in responsiveness. A, data combined from 30 oocytes (three separate preparations) injected with UTR-containing α4 [α4(u)) and β2 (β2(u)] nAChR messages in approximately equal amounts (1:1 ratio). The curve shows a Hill equation fit to the combined data with an EC_{50} value of 2.2 μM [1.9–2.6 μM confidence interval (CI)], Hill slope of 1.05 ± 0.06, and plateau of 105 ± 0.8%. Curves fit to individual data from each of 28 oocytes provided similar results; in the other two oocytes, data were insufficient for curve fitting. B, data are from seven oocytes injected with a 1:1 ratio α4 and β2 nAChR messages lacking UTR. The fitted curve is the sum of two independent Hill equations with EC_{50} values of 0.54 μM (0.086–3.41 μM CI) and 114 μM (90–145 μM CI), Hill slopes of 1.01 ± 0.35 and 1.39 ± 0.23, and plateaus of 13 ± 4 and 84 ± 6%. Data are shown as mean ± S.E.M. or mean ± 95% CI for EC_{50} values.

entially according to cell type or maturation. However, the α4(u) and β2(u) clones represent sequences that, because they contain partial or full UTR, are closer to the native mRNA that would be expressed in brain than are the clones without UTR. Thus, it was of interest to explore the pharmacology of the high- and low-sensitivity α4β2 nAChR with the

TABLE 2

Isolation of a high-sensitivity α4β2 subform using UTR-containing messages expressed in oocytes

Data for EC_{50} are shown as mean and 95% confidence interval. Data for the Hill coefficient (n_H) are shown as mean ± S.E.M. For α4(u)β2(u) 1:1 and α4β2(u) 1:5, the data were fit by the monophasic Hill equation. Otherwise, the data were better fit by a biphasic curve representing the sum of two independent Hill equations; the proportion of the high-affinity component was estimated from the plateau values of the two fitted components. Receptors were expressed from α4 and β2 messages lacking UTR and from other messages, designated α4(u) and β2(u), that contained the same coding regions plus 3′- and 5′-UTR segments.

Receptor	Ratio α:β	n	Acetylcholine				
			High-Sensitivity			Low-Sensitivity	
			EC_{50}	n_H	Proportion	EC_{50}	n_H
			μM		%	μM	
α4(u)β2(u)	1:1	30	2.2 (1.9–2.6)	1.05 ± 0.06	100		
α4β2	9:1	5	0.70 (0.4–1.2)	1.5 ± 0.3	15	130 (80–190)	1.2 ± 0.1
α4β2	1:1	7	0.54 (0.09–3.4)	1.0 ± 0.4	14	114 (90–145)	1.4 ± 0.2
α4β2	1:9	6	2.3 (0.41–13)	1.2 ± 0.3	35	120 (56–270)	1.4 ± 0.8
α4β2	1:20	3	1.2 (0.05–30)	0.9 ± 0.7	32	200 (71–540)	1.1 ± 0.6
α4β2	1:60	9	3.1 (1.1–8.4)	1.0 ± 0.3	38	160 (120–210)	1.7 ± 0.3
α4β2	1:120	5	2.0 (0.41–9.5)	1.1 ± 0.5	46	100 (50–210)	1.6 ± 0.8
α4β2	1:1	3	2.5 (0.22–29)	0.9 ± 0.5	24	140 (110–180)	2.0 ± 0.4
α4(u)β2	1:1	4	4.0 (0.30–55)	1.1 ± 0.6	21	200 (120–360)	1.5 ± 0.6
α4β2(u)	1:1	10	0.85 (0.16–4.6)	1.3 ± 1.1	39	120 (45–350)	1.1 ± 0.6
α4(u)β2(u)	1:1	3	2.1 (2.0–2.2)	1.15 ± 0.02	100		
α4β2	1:1	4	1.5 (0.56–4.2)	1.1 ± 0.4	16	180 (160–210)	1.5 ± 0.2
α4β2(u)	1:5	4	1.6 (1.2–2.3)	1.2 ± 0.2	100		

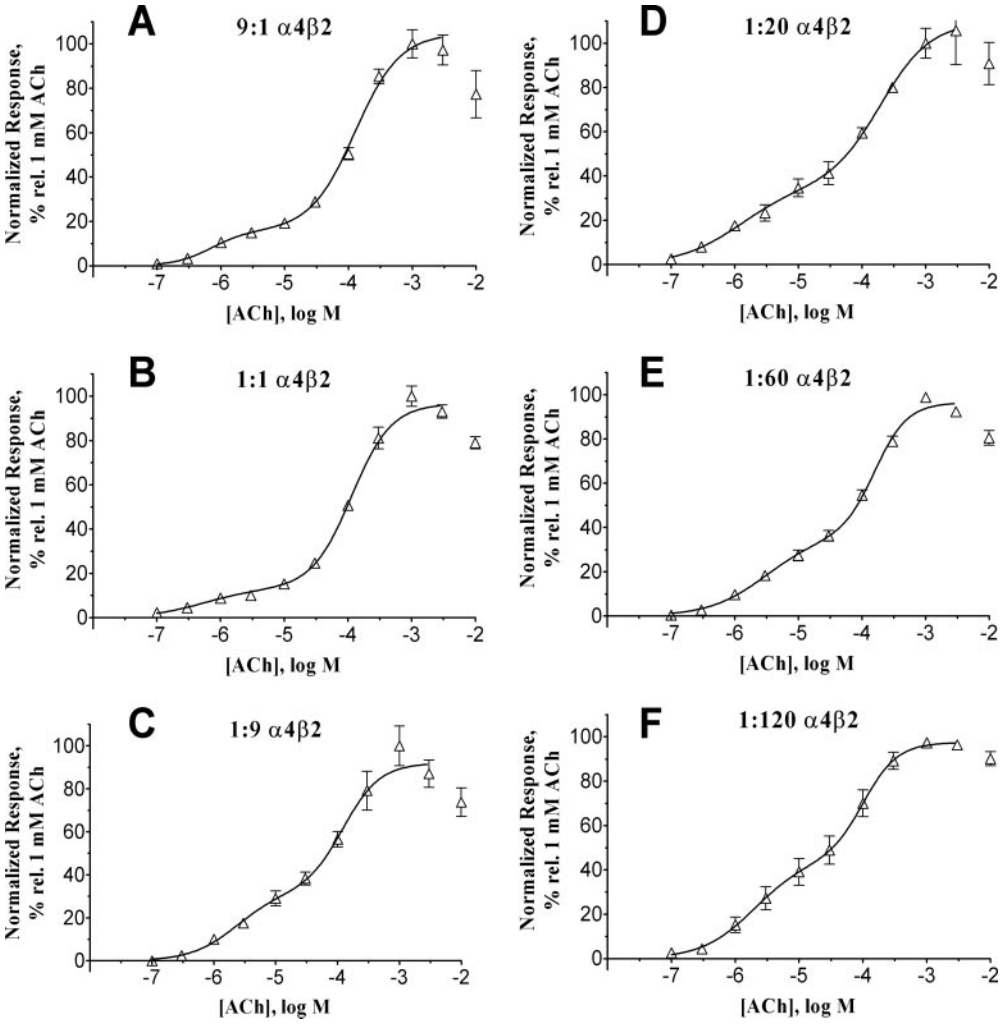

Fig. 3. Inability to isolate high- and low-sensitivity components by adjusting ratio of messages lacking UTR. Oocytes were injected with α4 and β2 subunit messages in various ratios according to nucleotide content. Volume and total nucleotide content were similar. Relative amounts injected were nine α4 to one β2 (A), one α4 to one β2 (B), one α4 to nine β2 (C), one α4 to 20 β2 (D), one α4 to 60 β2 (E), and one α4 to 120 β2 (F). Data for B are the same as in Fig. 2B and are reproduced here to facilitate comparison with other α4/β2 ratios. Concentration-response parameters, determined as in Fig. 2, are provided in Table 2.

aim of uncovering selective tools that could be used to elucidate the properties and physiological roles of the receptors.

Five antagonists were evaluated for their effects on high- and low-sensitivity forms of $\alpha4\beta2$. This was performed by using receptors expressed from $\alpha4(u)\beta2(u)$ to generate the high-sensitivity form alone, and from $\alpha4\beta2$ without UTR to generate mixed high- and low-sensitivity receptors. We were not able to express the low-sensitivity form alone. With both $\alpha4(u)\beta2(u)$ and $\alpha4\beta2$, antagonist IC_{50} values were measured against two concentrations of acetylcholine, 2 μM (near the high-sensitivity EC_{50}) and 200 μM (near the low-sensitivity EC_{50}). In the mixed sensitivity $\alpha4\beta2$ population, most (~97%) of the response to 2 μM acetylcholine should have been from the high-sensitivity $\alpha4\beta2$ subform, whereas for 200 μM acetylcholine most (~81%) of the response should have been from the low-sensitivity $\alpha4\beta2$ subform based upon concentration-response parameters shown in Table 2. The antagonist concentration-inhibition curves are shown in Figs. 7 and 8, and IC_{50} values are in Table 4.

Neither DHβE nor methyllycaconitine distinguished between the high-and low-sensitivity forms (Fig. 7). IC_{50} values were 3 to 6 nM for DHβE and 40 to 135 nM for methyllycaconitine under all conditions. In contrast, chlorisondamine, and to some extent mecamylamine and *d*-tubocurarine, seemed to be selective for the low-sensitivity form (Fig. 8). When 200 μM acetylcholine and the mixed sensitivity $\alpha4\beta2$ were used, the IC_{50} values were 0.2 μM for mecamylamine, 0.9 μM for *d*-tubocurarine, and 0.2 μM for chlorisondamine. When the isolated high-sensitivity form, $\alpha4(u)\beta2(u)$, and 2 μM acetylcholine were used, IC_{50} values were 8-, 5-, and 100-fold higher for mecamylamine, *d*-tubocurarine, and chlorisondamine, respectively.

Modulation of $\alpha4\beta2$ Subforms by Estradiol. 17β-Estradiol is a neuroactive steroid that has been found to potentiate human $\alpha4\beta2$, whereas inhibiting other nAChR (Nakazawa and Ohno, 2001; Paradiso et al., 2001; Curtis et al., 2002). Estradiol clearly potentiated the acetylcholine response at the high-sensitivity ferret $\alpha4(u)\beta2(u)$, as shown in Fig. 9. In the mixed sensitivity population, however, the potentiation was weaker. It was not clear whether this was due to a selective potentiation of the high-sensitivity subform or to a mixture of effects at both subforms.

Agonist Efficacy at $\alpha4\beta2$ Subforms. In rat brain, $\alpha4\beta2$ makes up the majority of the high-affinity binding sites for (−)-nicotine (Whiting et al., 1991; Flores et al., 1992). However, in the mixed sensitivity population generated from $\alpha4\beta2$ messages lacking UTR, the apparent potency and efficacy values for (−)-nicotine were similar to those for acetylcholine (Fig. 10). In the high-sensitivity populations generated from $\alpha4$ and $\beta2$ messages containing UTR, or $\alpha4$ message lacking UTR plus $\beta2$ message containing UTR (1:5 message ratio), (−)-nicotine was as potent as in the mixed sensitivity population, but its apparent efficacy was only 24% relative to acetylcholine.

In contrast, analogs of A-84543 (Lin et al., 2001) were found to be highly selective for the high-sensitivity $\alpha4\beta2$ subform, based upon efficacy determinations that used $\alpha4(u)\beta2(u)$ and $\alpha4\beta2$. For example, A-163554 was highly efficacious at the ferret high-sensitivity $\alpha4\beta2$ expressed from

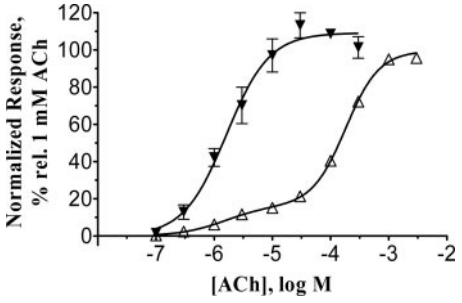

Fig. 5. Increased proportion of $\beta2$ message containing UTR eliminates the low-sensitivity component. Oocytes were injected with $\alpha4$ and $\beta2$ nAChR subunit messages both lacking UTR (open symbols) or $\alpha4$ nAChR subunit message lacking UTR plus $\beta2$ nAChR subunit message containing 3′- and 5′-UTR in a 1:5 nucleotide content ratio (closed symbols). Exclusive expression of the high-sensitivity component was obtained with an excess of UTR-containing $\beta2$ subunit relative to $\alpha4$ subunit. Data are from four oocytes of each type measured on the same day and concentration-response parameters are provided in Table 2.

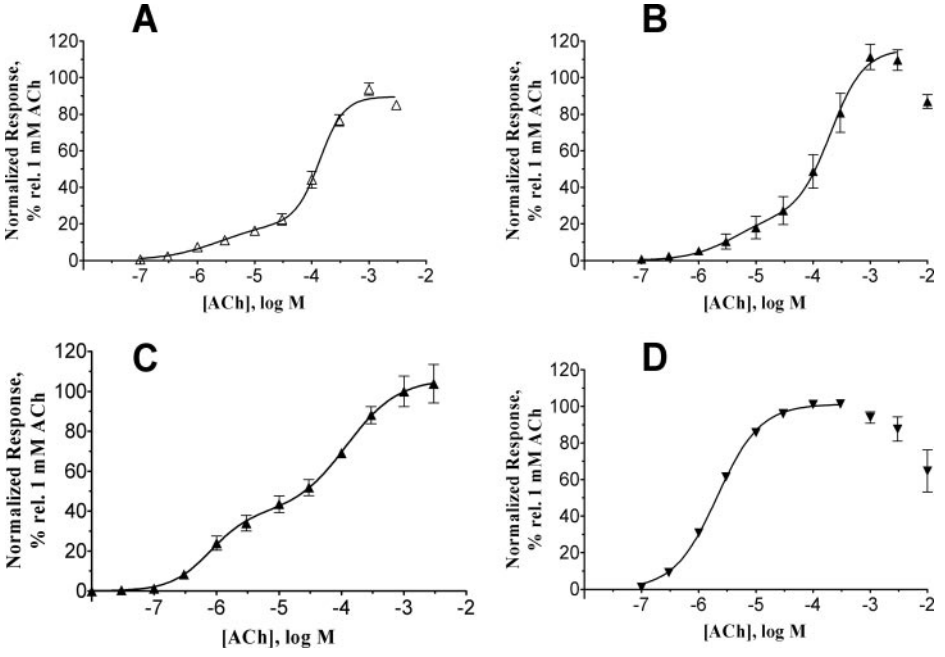

Fig. 4. Both $\alpha4$- and $\beta2$-UTR contribute to exclusive expression of the high-sensitivity $\alpha4\beta2$ nAChR. Oocytes were injected with equal amounts of $\alpha4$ subunit message without or with 3′- and 5′-UTR plus $\beta2$ subunit message without or with 3′- and 5′-UTR. Data are shown for $\alpha4\beta2$ nAChR from $\alpha4$ and $\beta2$ messages both lacking UTR (A), $\alpha4(u)\beta2$ nAChR from $\alpha4$ message containing 3′- and 5′-UTR and $\beta2$ message lacking UTR (B), $\alpha4\beta2(u)$ nAChR from $\alpha4$ message lacking UTR and $\beta2$ message containing 3′- and 5′-UTR (C), and $\alpha4(u)\beta2(u)$ nAChR from $\alpha4$ and $\beta2$ messages both containing 3′- and 5′-UTR (D). Concentration-response parameters, determined as in Fig. 2, are provided in Table 2.

UTR-containing $\alpha4$(u)$\beta2$(u) but seemed as if it were a partial agonist in the mixed high- and low-sensitivity populations expressed from $\alpha4\beta2$ lacking UTR (Fig. 11). Likewise, A-162035 (Fig. 12) and A-168939 (Fig. 13) were, in comparison with acetylcholine, full agonists at $\alpha4$(u)$\beta2$(u) but seemingly partial agonists in the mixed sensitivity $\alpha4\beta2$ population. These compounds seem to selectively activate the high-sensitivity $\alpha4\beta2$ response, thus producing an apparent partial response from oocytes expressing low- as well as high-sensitivity $\alpha4\beta2$.

A-163554 and A-168939 were somewhat less efficacious at $\alpha4\beta2$ than anticipated from their efficacy at $\alpha4$(u)$\beta2$(u) and assumption of 15% high-sensitivity subform in the mixed sensitivity $\alpha4\beta2$ population. This may be due to functional differences between high-sensitivity subforms from $\alpha4$(u)$\beta2$(u) compared with $\alpha4\beta2$ or to variance in the relative amount of the high-sensitivity subform expressed from $\alpha4\beta2$. A-162035 seemed more efficacious than the other analogs at $\alpha4\beta2$, probably because of some activity at low-sensitivity as well as high-sensitivity $\alpha4\beta2$.

Agonist Efficacy at Native $\alpha4\beta2$. A-162035 and A-168939 were used to test whether receptors similar to the high-sensitivity $\alpha4\beta2$ subform could be expressed in brain. A-162035 (Fig. 14A) and A-168939 (Fig. 14B) each stimulated $\alpha4\beta2$-mediated $^{86}Rb^+$ flux in mouse thalamic synaptosomes. The EC_{50} values for $^{86}Rb^+$ flux (see figure legend) were remarkably similar to the EC_{50} values determined by using ferret $\alpha4$(u)$\beta2$(u), despite the differences in species and assay types. Furthermore, maximal responses to A-162035 and A-168939 were nearly as large as the response to 10 μM (−)-nicotine, which has been shown to be selective for the high-sensitivity $\alpha4\beta2$ nAChR response in mouse thalamus (Marks et al., 1999, 2004). Figure 14C also shows the thalamic synaptosome response to 10 μM (−)-nicotine in relation to the biphasic acetylcholine concentration-response relationship. Responses to 10 to 100 μM A-162035 and A-168939 were essentially completely blocked by 2 μM DHβE, which also has been shown to be selective for the high-sensitivity $\alpha4\beta2$ nAChR in this assay.

In mouse brain synaptosomes, A-168939 seemed to be

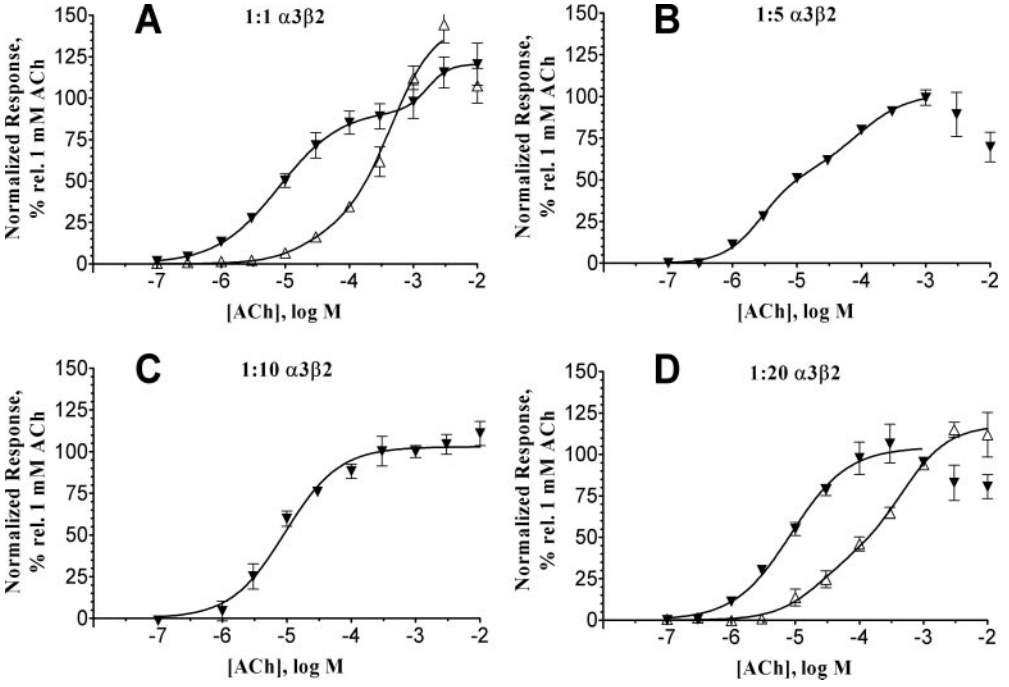

Fig. 6. Expression of high-sensitivity $\alpha3\beta2$ nAChR with the use of UTR-containing message. Oocytes were injected with $\alpha3$ subunit message lacking UTR plus $\beta2$ subunit message either lacking UTR (open symbols) or containing 3′- and 5′-UTR (closed symbols). The $\alpha3/\beta2$ nucleotide content ratios were 1:1 (A), 1:5 (B), 1:10 (C), and 1:20 (D). Concentration-response parameters are provided in Table 3.

TABLE 3

Potencies for acetylcholine at high-sensitivity and mixed-sensitivity ferret $\alpha3\beta2$

For $\alpha3\beta2$(u) 1:10 and 1:20 ratios, the data for acetylcholine concentrations up to 1 mM were fit with a monophasic Hill equation. Other data were fit with a biphasic curve, as in Table 2. Hill coefficients for $\alpha3\beta2$ 1:20 were constrained to fit the data. Receptors were expressed from $\alpha3$ message lacking UTR and from $\beta2$ messages lacking ($\beta2$) or containing [($\beta2$(u)] 3′- and 5′-UTR segments.

Receptor and $\alpha:\beta$ Ratio	n	Acetylcholine				
		High-Sensitivity			Low-Sensitivity	
		EC_{50}	n_H	Proportion	EC_{50}	n_H
		μM		%	μM	
$\alpha3\beta2$						
1:1	15	25 (7–88)	1.2 ± 0.2	15	450 (380–540)	1.3 ± 0.1
1:20	6	23 (7–81)	1.2	34	480 (200–1100)	1.2
$\alpha3\beta2$(u)						
1:1	13	8.1 (7.1–9.1)	0.89 ± 0.03	77	1700 (1300–2100)	2.6 ± 0.3
1:5	3	2.8 (1.4–5.6)	1.5 ± 0.4	52	88 (29–260)	1.1 ± 0.6
1:10	9	9.4 (6.6–13)	1.0 ± 0.2	100		
1:20	8	8.6 (6.0–12)	0.98 ± 0.14	100		

slightly more efficacious than A-162035, whereas the reverse was found by using ferret $\alpha4(u)\beta2(u)$ expressed in oocytes. Nevertheless, the synaptosome data for A-162035 and A-168939 agree well with the oocyte $\alpha4(u)\beta2(u)$ data in contrast to the mixed sensitivity $\alpha4\beta2$ data. Overall, the results are consistent with the idea that the high-sensitivity $\alpha4\beta2$ subform is expressed in brain and that the agonists A-162035 and A-168939 selectively activate that receptor.

Higher concentrations of A-162035 ($\geq$3 μM) and A-168939 ($\geq$10 μM) seemed to inhibit the synaptosomal response to the same compounds (Fig. 14), possibly because of nAChR channel block or desensitization. A similar effect, at somewhat higher concentrations, was observed with ferret $\alpha4\beta2$ expressed in oocytes (Figs. 12 and 13). High concentrations of acetylcholine and nicotine also can produce an inhibitory effect (Figs. 2–10). The mechanism of this inhibition was not investigated.

Discussion

The main findings in this study are that 1) ferret $\alpha4\beta2$ nAChR could be expressed exclusively in the high-sensitivity form only from UTR-containing message; 2) the principal determinant seems to be in the $\beta2$-UTR, although $\alpha4$-UTR also may contribute; 3) a high-sensitivity form of $\alpha3\beta2$ also could be exclusively expressed with UTR-containing $\beta2$; 4) high- and low-sensitivity $\alpha4\beta2$ could be distinguished pharmacologically by certain antagonists and agonists as well as by the potency of the neurotransmitter acetylcholine; and 5) agonists selective for the high-sensitivity $\alpha4\beta2$ subform were

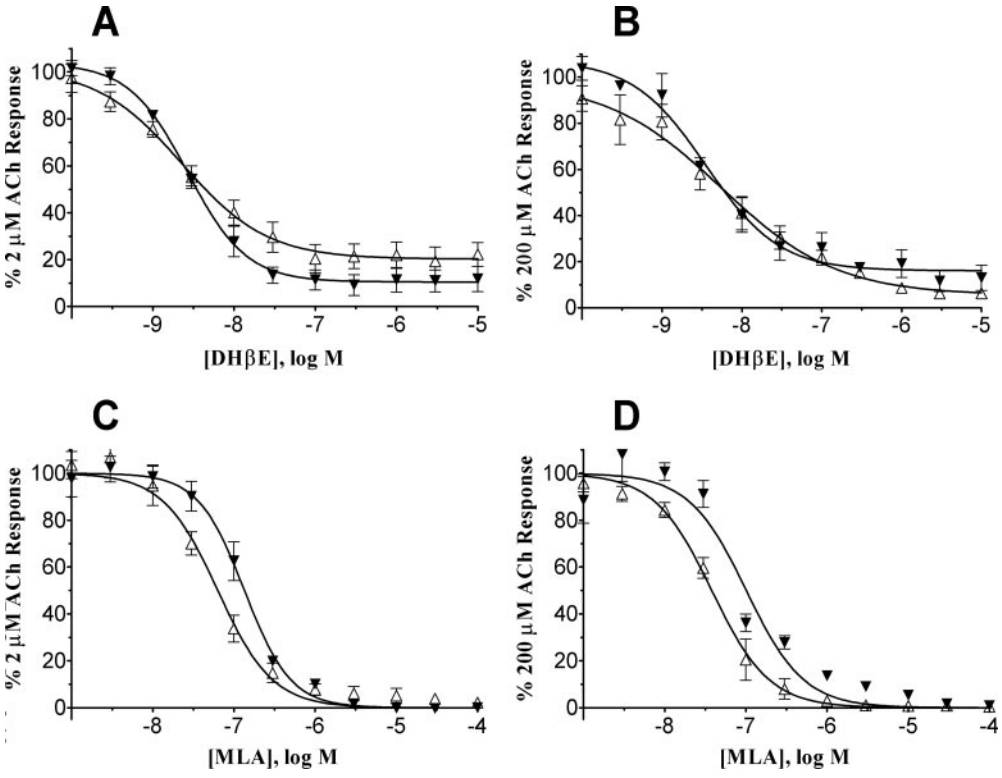

Fig. 7. Antagonists dihydro-β-erythroidine and methyllycaconitine are not selective for high- versus low-sensitivity $\alpha4\beta2$ nAChR. Concentration-inhibition data for dihydro-β-erythroidine (DHβE, A and B) and methyllycaconitine (MLA, C and D) were obtained in oocytes expressing a mixture of high- and low-sensitivity $\alpha4\beta2$ nAChR (open symbols) and in oocytes expressing exclusively the high-sensitivity $\alpha4\beta2$ nAChR from UTR-containing $\alpha4$ and $\beta2$ messages (closed symbols). For both mixed sensitivity and exclusively high-sensitivity nAChR, inhibition was measured against 2 μM acetylcholine (A and C) and 200 μM acetylcholine (B and D). Concentration-inhibition parameters are provided in Table 4.

TABLE 4

Antagonist potencies at ferret $\alpha4\beta2$ and at the high-sensitivity form expressed from UTR-containing $\alpha4(u)$ and $\beta2(u)$

Concentration-inhibition curves projected to 100% inhibition for methyllycaconitine, mecamylamine, *d*-tubocurarine, and chlorisondamine, but not for DHβE. For DHβE acting on $\alpha4\beta2$ the projected maximal inhibition was 80 $\pm$ 3% with 2 μM acetylcholine and 95 $\pm$ 4% with 200 μM acetylcholine, whereas these values at $\alpha4(u)\beta2(u)$ were 90 $\pm$ 2% with 2 μM acetylcholine and 84 $\pm$ 3% with 200 μM acetylcholine.

Antagonist	$\alpha4\beta2$			$\alpha4(u)\beta2(u)$		
	IC_{50}	n_H	n	IC_{50}	n_H	n
	μM			μM		
2 μM Acetylcholine						
DHβE	0.0023 (0.0010–0.0050)	0.83 $\pm$ 0.20	3	0.0027 (0.0020–0.0036)	1.20 $\pm$ 0.18	3
MLA	0.063 (0.049–0.081)	1.29 $\pm$ 0.18	3	0.13 (0.11–0.16)	1.52 $\pm$ 0.18	3
Mecamylamine	0.58 (0.27–1.26)	0.65 $\pm$ 0.14	3	2.0 (1.2–3.3)	0.72 $\pm$ 0.12	3
d-Tubocurarine	110 (11–1200)	0.41 $\pm$ 0.30	3	4.7 (3.2–7.0)	0.78 $\pm$ 0.11	4
Chlorisondamine	0.52 (0.34–0.80)	0.63 $\pm$ 0.08	5	18 (13–26)	1.00 $\pm$ 0.16	5
200 μM Acetylcholine						
DHβE	0.0056 (0.0021–0.015)	0.60 $\pm$ 0.15	3	0.0036 (0.0020–0.0062)	0.95 $\pm$ 0.21	3
MLA	0.038 (0.032–0.045)	1.23 $\pm$ 0.11	3	0.101 (0.073–0.139)	1.23 $\pm$ 0.21	3
Mecamylamine	0.24 (0.21–0.28)	0.90 $\pm$ 0.05	6	3.81 (2.67–5.64)	1.10 $\pm$ 0.21	6
d-Tubocurarine	0.92 (0.69–1.23)	0.75 $\pm$ 0.07	5	49.7 (8.8–280)	0.39 $\pm$ 0.13	3
Chlorisondamine	0.18 (0.17–0.20)	1.14 $\pm$ 0.05	6	3.3 (2.9–3.7)	0.84 $\pm$ 0.03	2

active at native $\alpha4\beta2$ in mouse brain as well as at recombinant ferret $\alpha4\beta2$.

It has been reported that the proportion of high-sensitivity $\alpha4\beta2$ could be increased by increasing the amount of $\beta2$ message (Zwart and Vijverberg, 1998) or by prolonged exposure to low concentrations of nicotine or reduced temperature (Buisson and Bertrand, 2001; Nelson et al., 2003). Zhou et al. (2003) also revealed biphasic concentration-response curves and monophasic high-sensitivity concentration-response curves for acetylcholine depending upon the $\alpha4$-$\beta2$ concatamer arrangement or the addition of free $\beta2$ message. These studies have suggested that high- and low-sensitivity components may correspond to $\alpha4_{(2)}\beta2_{(3)}$, and $\alpha4_{(3)}\beta2_{(2)}$ pentamers, respectively.

When ferret messages were used, increasing the relative amount of $\beta2$ message seemed to increase the proportion of high-sensitivity $\alpha4\beta2$, similar to previous reports with $\alpha4\beta2$ from other species. Zwart and Vijverberg (1998) also observed mixed high- and low-sensitivity $\alpha4\beta2$, even with the 1:9 message ratio. However, exclusive expression of the high-sensitivity $\alpha4\beta2$ subform (or the high-sensitivity $\alpha3\beta2$ subform) could be achieved by using ferret $\beta2$ message contain-

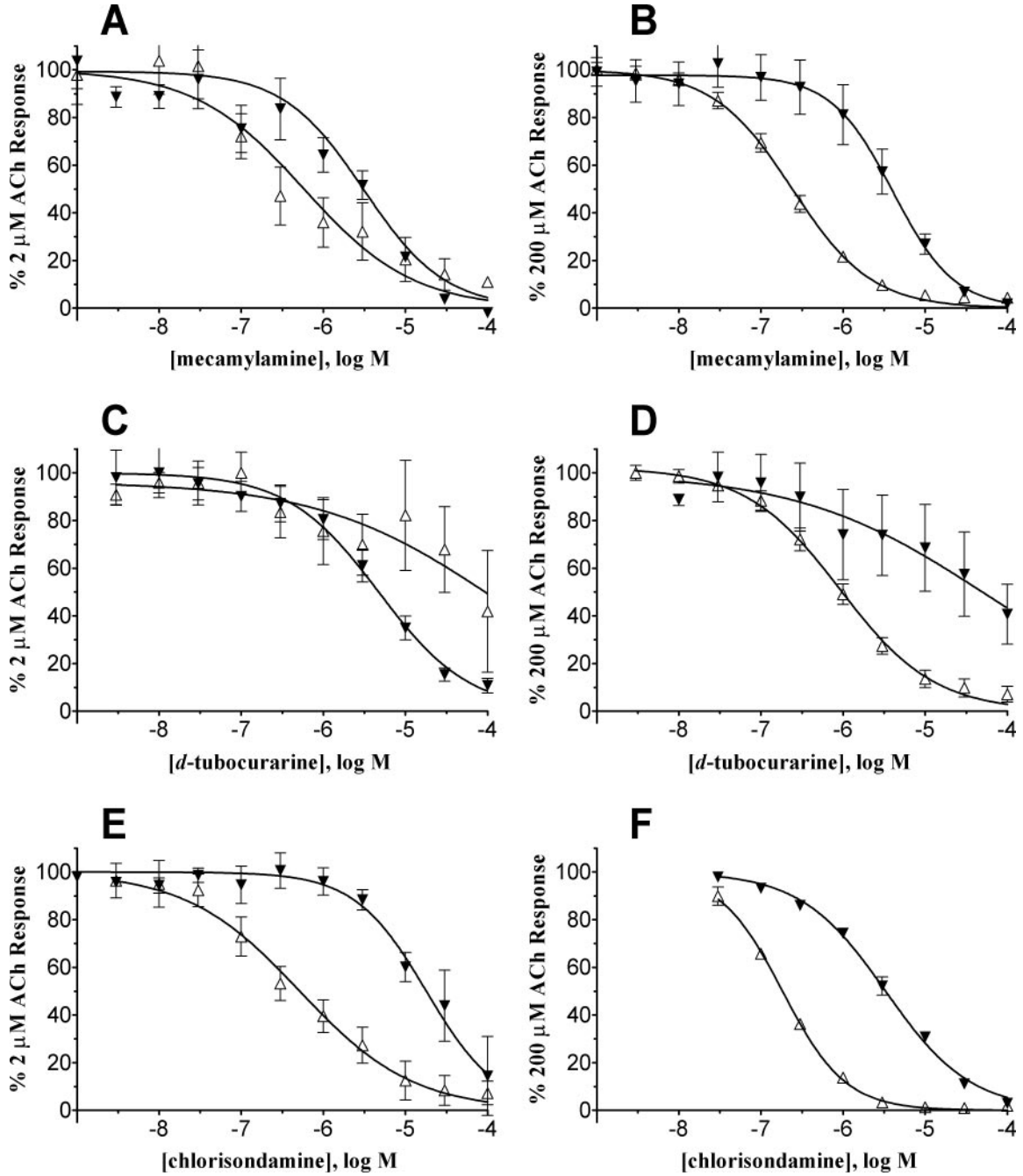

Fig. 8. Three antagonists seem selective for the low-sensitivity $\alpha4\beta2$ component. Concentration-inhibition data for mecamylamine (A and B), *d*-tubocurarine (C and D), and chlorisondamine (E and F) were obtained as described in Fig. 7. Data are shown for oocytes expressing mixed-sensitivity $\alpha4\beta2$ nAChR (open symbols) and high-sensitivity $\alpha4\beta2$ nAChR from UTR-containing $\alpha4$ and $\beta2$ messages (closed symbols). For both mixed and high-sensitivity $\alpha4\beta2$, the inhibition of responses to 2 μM acetylcholine (A, C, and E) and 200 μM acetylcholine (B, D, and F) was measured. Concentration-inhibition parameters are provided in Table 4.

ing UTR, but not by using messages lacking UTR. It is assumed that the same $\alpha4$ and $\beta2$ proteins are expressed with or without UTR. High-sensitivity ferret $\alpha4\beta2$ expression may be particularly dependent upon the presence of UTR for message stability or protein translation, and at very low $\alpha4/\beta2$ ratios without UTR the small amount of $\alpha4$ may limit the ability to detect functional $\alpha4\beta2$ expression. Short UTR segments in the human messages (Nelson et al., 2003; Zhou et al., 2003) and possibly rat messages (Zwart and Vijverberg, 1998) used in previous reports also may have influenced high-sensitivity $\alpha4\beta2$ expression; this remains to be investigated. In addition, it should be noted that the $\beta2$

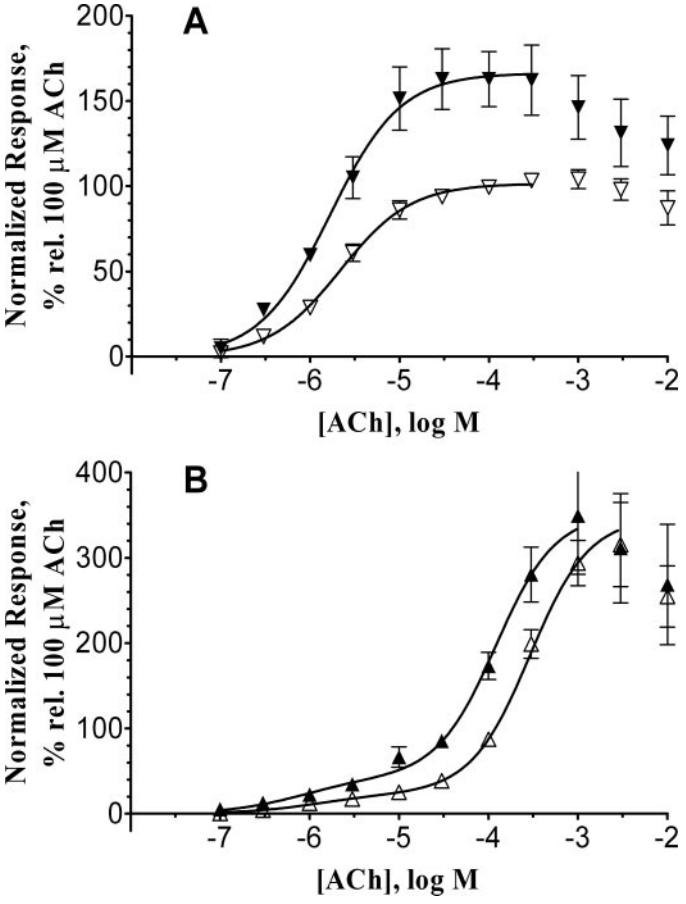

Fig. 9. Estradiol potentiation of $\alpha4\beta2$ nAChR. Acetylcholine concentration-response data in the absence and presence of 10 μM 17β-estradiol were obtained from three oocytes expressing high-sensitivity $\alpha4\beta2$ nAChR by using UTR-containing messages and from three oocytes expressing mixed sensitivity $\alpha4\beta2$ nAChR by using messages lacking UTR. The concentration of acetylcholine in the reference control was 100 μM for both mixed and high-sensitivity $\alpha4\beta2$ oocytes. A, at high-sensitivity $\alpha4\beta2$ nAChR, estradiol increased the maximal response by 64%. In the absence (open symbols) and presence (closed symbols) of estradiol, respectively, the acetylcholine EC_{50} values were 2.2 μM (1.8–2.6 μM CI) and 1.7 μM (1.0–2.7 μM CI), Hill slopes were 1.10 $\pm$ 0.10 and 1.10 $\pm$ 0.24, and plateaus were 102 $\pm$ 2 and 166 $\pm$ 8% relative to 100 μM acetylcholine in the absence of estradiol. B, estradiol also potentiated acetylcholine responses in the mixed sensitivity population, but overall the effect was smaller than with high-sensitivity $\alpha4\beta2$ alone. Concentration-response parameters were determined as for other data, except that Hill slopes were constrained to be shared between data with and without estradiol to fit biphasic curves. For the high-sensitivity component, in the absence (open symbols) and presence (closed symbols) of estradiol, respectively, the acetylcholine EC_{50} values were 1.2 μM (0.59–2.6 μM CI) and 0.82 μM (0.31–2.2 μM CI), Hill slopes were 1.06 $\pm$ 0.18, and plateaus were 24 $\pm$ 3 and 43 $\pm$ 8% relative to control 100 μM acetylcholine in the absence of estradiol. For the low-sensitivity component in the absence and presence of estradiol, respectively, the acetylcholine EC_{50} values were 290 μM (210–400 μM CI) and 120 μM (77–190 μM CI), Hill slopes were 1.31 $\pm$ 0.13, and plateaus were 320 $\pm$ 30 and 310 $\pm$ 40% relative to control 100 μM acetylcholine in the absence of estradiol.

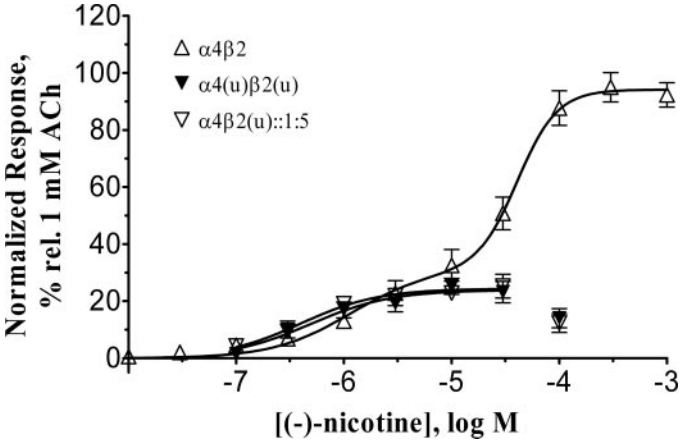

Fig. 10. Effects of nicotine in the high- and mixed sensitivity $\alpha4\beta2$ populations. Responses to (−)-nicotine were normalized to control 1 mM acetylcholine in each oocyte. In the mixed sensitivity $\alpha4\beta2$ population generated from messages lacking UTR (n = 15), (−)-nicotine activated both high- and low-sensitivity components. The fitted curve shows EC_{50} values of 1.1 μM (0.52–2.5 μM CI) and 41 μM (31–54 μM CI), Hill slopes of 1.2 (constrained) and 2.3 $\pm$ 0.78, and plateaus of 31 $\pm$ 5 and 63 $\pm$ 7% relative to 1 mM acetylcholine. However, in the high-sensitivity populations generated with the use of $\alpha4$ and $\beta2$ messages both containing UTR [$\alpha4(u)\beta2(u)$, 1:1 ratio; n = 4], the apparent efficacy of (−)-nicotine was less. The EC_{50} value was 0.49 μM (0.22–1.1 μM CI), the Hill slope was 1.16 $\pm$ 0.43, and the plateau was 24 $\pm$ 2%. Similar results were obtained with the use of $\alpha4$ message lacking UTR and $\beta2$ message containing UTR [$\alpha4\beta2(u)$ 1:5; n = 3], with which the (−)-nicotine EC_{50} value was 0.40 μM (0.24–0.66 μM CI), the Hill slope was 1.20 $\pm$ 0.30, and the plateau was 24 $\pm$ 1%. Fitted curves for both high-sensitivity subforms are shown in the figure but are essentially overlapping.

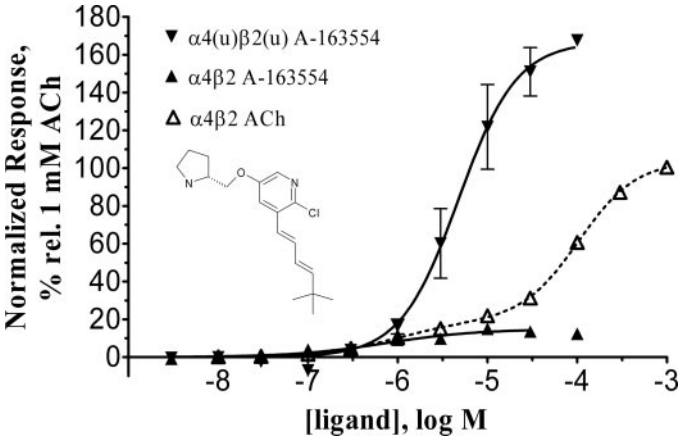

Fig. 11. Selectivity of A-163554 for high-sensitivity $\alpha4\beta2$. Responses are shown for A-163554 acting on the high-sensitivity receptor expressed from $\alpha4$ and $\beta2$ messages containing UTR [$\alpha4(u)\beta2(u)$; n = 3] and the mixed sensitivity population expressed from messages lacking UTR ($\alpha4\beta2$; n = 3). As an additional control, full concentration-response relationships for acetylcholine were determined in the same three $\alpha4\beta2$ oocytes exposed to A-163554. The fitted curves for A-163554 at $\alpha4(u)\beta2(u)$ show an EC_{50} value of 4.8 μM (3.1–7.3 μM CI), Hill slope of 1.34 $\pm$ 0.28 and plateau of 167 $\pm$ 12% relative to 1 mM acetylcholine. When mixed sensitivity $\alpha4\beta2$ was used, the responses to A-163554 again were fitted well by a monophasic concentration-response curve, with an EC_{50} value of 0.74 μM (0.23–2.4 μM CI), Hill slope of 0.85 $\pm$ 0.30, and plateau of 15 $\pm$ 2% relative to 1 mM acetylcholine. The same $\alpha4\beta2$ oocytes demonstrated a biphasic concentration response to acetylcholine with EC_{50} values of 0.92 μM (0.39–2.2 μM CI) and 105 μM (92–119 μM CI), Hill slopes of 1.05 $\pm$ 0.26 and 1.35 $\pm$ 0.13, and plateaus of 19 $\pm$ 3 and 85 $\pm$ 5% relative to 1 mM acetylcholine.

TM3-TM4 cytoplasmic loop is shorter in ferret $\beta 2$ than in human and rat $\beta 2$, largely because of two sequences of amino acids, one of eight amino acids located 38 residues upstream from TM4 and the other of 13 amino acids located 15 residues further upstream. It is possible that $\alpha 4\beta 2$ or $\alpha 3\beta 2$ assembly could be affected by the shorter loop. However, next to TM3 and TM4 the critical "proximal" amino acids of the cytoplasmic loop (Kuo et al., 2005) are identical in ferret, human, and rat.

The ferret $\alpha 4$-UTR also seemed to have an effect on exclusive expression of the high-sensitivity form. It is notewoerthy that the 5′ $\alpha 4$-UTR contains an open reading frame (ORF) that seems to be conserved among ferret, rat, and human (Supplemental Data). Examples of an upstream ORF affecting downstream translation are known (Morris and Geballe, 2000). However, there is no direct evidence that the $\alpha 4$ 5′ ORF affects coding sequence translation or is itself translated.

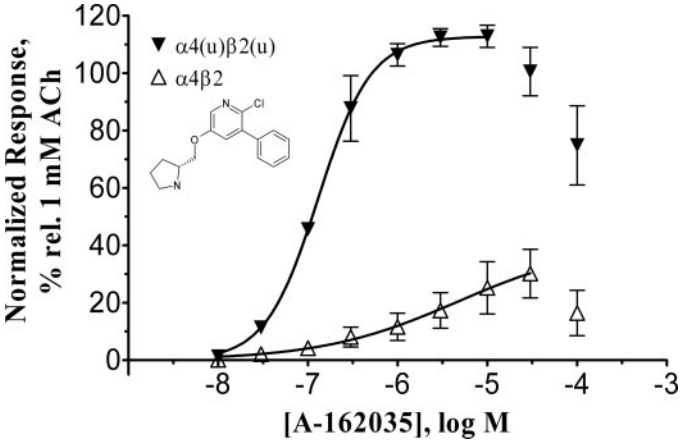

Fig. 12. Selectivity of A-162035 for high-sensitivity $\alpha 4\beta 2$. Responses are shown for A-162035 acting on the high-sensitivity receptor expressed from $\alpha 4$ and $\beta 2$ messages containing UTR [$\alpha 4(u)\beta 2(u)$; $n = 3$] and the mixed sensitivity population expressed from messages lacking UTR ($\alpha 4\beta 2$; $n = 4$). The fitted curves for A-162035 at $\alpha 4(u)\beta 2(u)$ show an EC_{50} value of 0.13 μM (0.11–0.16 μM CI), Hill slope of 1.49 ± 0.20, and plateau of 113 ± 3% relative to 1 mM acetylcholine. When $\alpha 4\beta 2$ mixed sensitivity receptors were used, A-162035 did not have a clear biphasic concentration-response relationship, but the low Hill slope suggested that the compound may act upon more than one receptor type. The fitted curve shown reflects an EC_{50} value of 4.6 μM (0.02–108 μM CI), Hill slope of 0.57 ± 0.35, and plateau of 41 ± 26% relative to 1 mM acetylcholine.

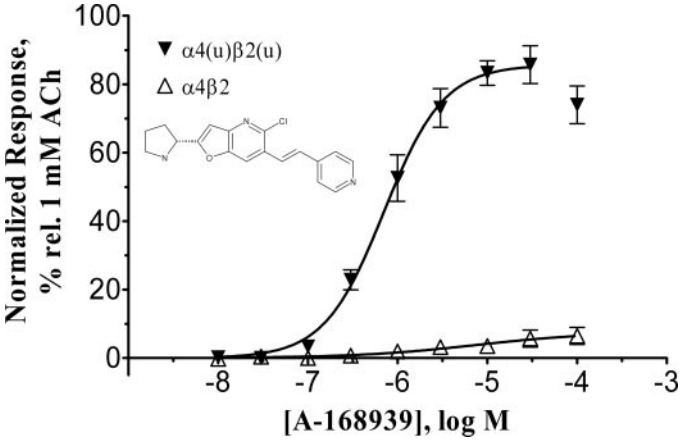

Fig. 13. Selectivity of A-168939 for high-sensitivity $\alpha 4\beta 2$. Responses are shown for A-168939 acting on the high-sensitivity receptor expressed from $\alpha 4$ and $\beta 2$ messages containing UTR [$\alpha 4(u)\beta 2(u)$; $n = 6$] and the mixed sensitivity population expressed from messages lacking UTR ($\alpha 4\beta 2$; $n = 6$). The fitted curve for A-168939 at $\alpha 4(u)\beta 2(u)$ shows an EC_{50} value of 0.71 μM (0.54–0.93 μM CI), Hill slope of 1.31 ± 0.18, and plateau of 86 ± 3% relative to 1 mM acetylcholine. A-168939 had little effect at $\alpha 4\beta 2$ mixed-sensitivity receptors. The fitted curve reflects an EC_{50} value of 7.5 μM (0.16–360 μM CI), Hill slope of 0.65 ± 0.40, and plateau of 8 ± 4% relative to 1 mM acetylcholine. The low Hill slope may reflect activation of more than one receptor type or simply the difficulty in resolving the concentration-response relationship for such small responses.

For $\alpha 3\beta 2$, a wide range of acetylcholine EC_{50} values have been reported, from 1.2 to 443 μM (Gerzanich et al., 1995; Chavez-Noriega et al., 1997; Colquhoun and Patrick, 1997), and Covernton and Connolly (2000) suggested a biphasic $\alpha 3\beta 2$ concentration-response. Using ferret $\beta 2$ with UTR, we demonstrated that $\alpha 3\beta 2$ as well as $\alpha 4\beta 2$ indeed could exhibit a biphasic concentration-response relationship for acetylcholine. Furthermore, the high-sensitivity $\alpha 3\beta 2$ subform could be exclusively expressed by using a 1:20 ratio of $\alpha 3/\beta 2(u)$. To our knowledge, this is the first report that decreasing $\alpha 3/\beta 2$ message ratio influences $\alpha 3\beta 2$ sensitivity to acetylcholine, and the first exclusive expression of the high-sensitivity subform.

In many studies with recombinant $\alpha 4\beta 2$ nAChR, higher EC_{50} forms seem to predominate (Gopalakrishnan et al., 1996; Chavez-Noriega et al., 2000; Houlihan et al., 2001; Nelson et al., 2003), whereas predominant low EC_{50} values are observed in other forms (Bertrand et al., 1990; Buisson et al., 1996; Kuryatov et al., 1997). In CNS, $\alpha 4\beta 2$ nAChR demonstrate low EC_{50} corresponding to high-sensitivity $\alpha 4\beta 2$ (Alkondon and Albuquerque, 1993, 1995; Marks et al., 1993, 1999; Marszalec et al., 1999). Such variances raise questions regarding the extension of recombinant nAChR pharmacology to native nAChR.

To identify compounds that may be useful in evaluating the physiological roles of high- and low-sensitivity $\alpha 4\beta 2$, several antagonists and agonists were evaluated for selectivity. These experiments used $\alpha 4(u)\beta 2(u)$ to express exclusively the high-sensitivity subform, and $\alpha 4\beta 2$ to express a mixture of high- and low-sensitivity subforms. Although the antagonists DHβE and methyllycaconitine were not selective between $\alpha 4\beta 2$ subforms, chlorisondamine, mecamylamine and *d*-tubocurarine were somewhat selective for the low-sensitivity $\alpha 4\beta 2$ subform. Our results with *d*-tubocurarine were generally similar to those of Zwart and Vijverberg (1998), who used another species' $\alpha 4\beta 2$. Both studies found low IC_{50} (0.5–1 μM) and low Hill coefficient (n_H) (0.71–0.77) for 1:1 $\alpha 4\beta 2$ and high concentrations of acetylcholine (200 or 300 μM), and both found similar values (2–5 μM IC_{50} values, 0.67–0.78 Hill coefficients) for high sensitivity $\alpha 4\beta 2$ and lower concentrations of acetylcholine (2 or 10 μM). With 1:9 $\alpha 4\beta 2$ and 300 μM acetylcholine, Zwart and Vijverberg (1998) observed a biphasic concentration-inhibition curve, although it is not clear to what extent this was due to *d*-tubocurarine properties or the mixture of low- and high-sensitivity $\alpha 4\beta 2$ obtained with the 1:9 ratio. In our experiments with $\alpha 4(u)\beta 2(u)$ and 200 μM acetylcholine or 1:1 $\alpha 4\beta 2$ and 2 μM acetylcholine, we observed high IC_{50} values (50–100 μM) and low Hill coefficients (0.39–0.41), possibly reflecting an unresolved combination of low and high potencies for *d*-tubocurarine. The different potencies of *d*-tubocurarine at $\alpha 4\beta 2$ may reflect differences between high- and low-sensitivity $\alpha 4\beta 2$ receptors, differences between the two binding sites in each receptor, or different mechanisms of inhibition such as binding site displacement and channel block.

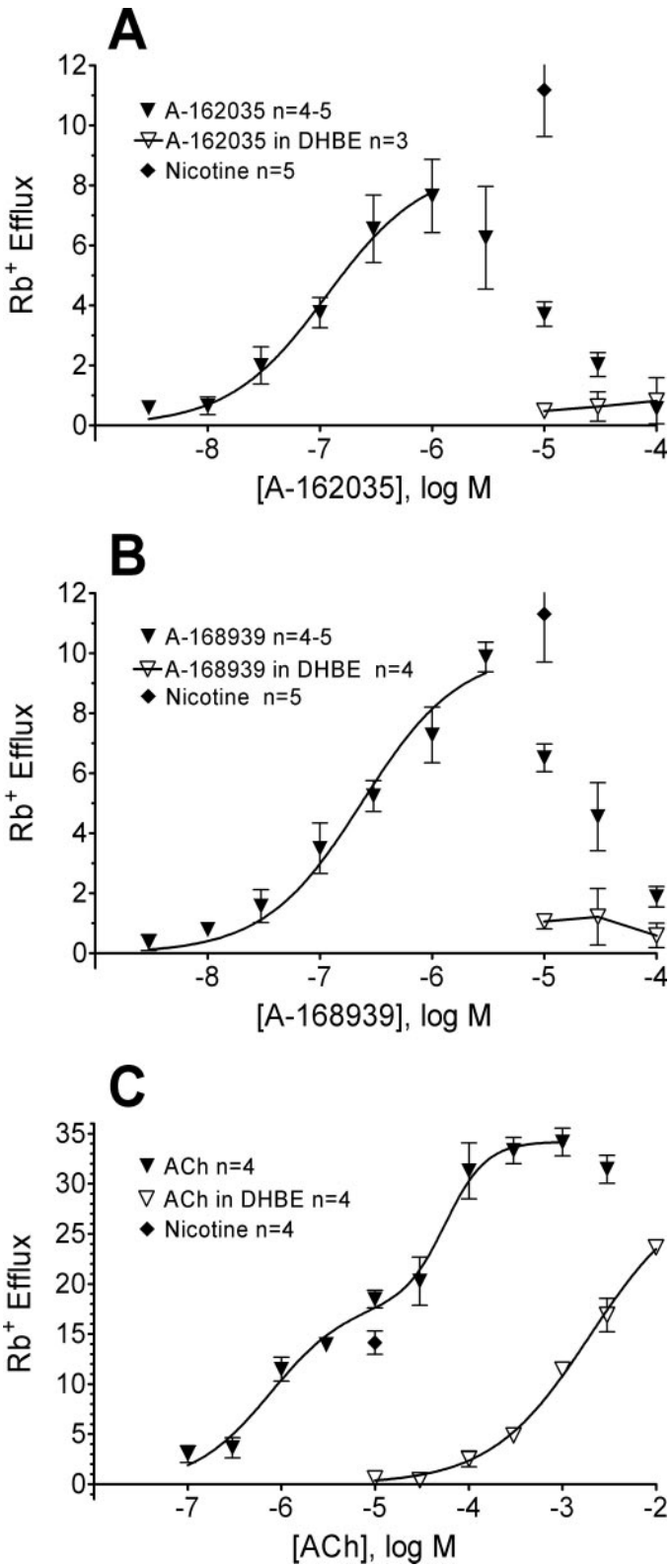

Fig. 14. Activation of native $\alpha4\beta2$ nAChR by A-162035 and A-168939. Mouse thalamic synaptosome Rb^+ flux was measured in response to A-162035, A-168939, and acetylcholine in the absence and presence of DHβE. Graphs also show the response to 10 μM (−)-nicotine (♦) measured in each experiment as a positive control for high-sensitivity $\alpha4\beta2$. A, concentration-response for A-162035 in the absence (▲; n = 4–5 each data point) and presence (△; n = 3) of 2 μM DHβE. The fitted curve for A-162035 shows an EC_{50} value of 0.12 μM (0.039–0.34 μM CI), Hill slope of 1.00 ± 0.33, and plateau of 8 ± 2% relative to the maximal response. B, concentration-response for A-168939 in the absence (▲; n = 4–5 each data point) and presence (△; n = 3) of 2 μM DHβE. The fitted curve for A-168939 shows an EC_{50} value of 0.24 μM (0.11–0.50 μM CI), Hill slope of 1.00 ± 0.24, and plateau of 10 ± 1% relative to the maximal response. C, for comparison, concentration-response for acetylcholine in the absence (▲; n = 4) and presence (△; n = 4) of 2 μM DHβE. Note the change in ordinate and abscissa scales compared with A and B. The concentration-response curve for acetylcholine was biphasic in the absence of DHβE. The fitted curve shows a high-sensitivity component with EC_{50} value of 0.77 μM (0.24–2.4 μM CI), Hill slope of 1.06 ± 0.40, and plateau of 18 ± 4% and a low-sensitivity component with EC_{50} value of 57 μM (28–115 μM CI), Hill slope of 2.00 ± 0.97, and plateau of 16 ± 4%.

In addition to antagonists selective for low-sensitivity $\alpha4\beta2$, agonists displaying efficacy selective for high-sensitivity $\alpha4\beta2$ could be identified. Analogs of A-84543 (Lin et al., 2001) seemed to activate predominantly high-sensitivity $\alpha4\beta2$. A-163554, A-162035, and A-168939 were full agonists at the high-sensitivity $\alpha4(u)\beta2(u)$ subform. In contrast, these compounds had the appearance of partial agonists in the mixed sensitivity $\alpha4\beta2$ population expressed from message lacking UTR, to an extent consistent with high efficacy at the high-sensitivity component and low efficacy at the low-sensitivity component.

To determine whether such compounds could activate native $\alpha4\beta2$, the effect on $^{86}Rb^+$ flux in mouse brain thalamic synaptosomes was measured under conditions selective for the $\alpha4\beta2$ component. A-162035 and A-168939 stimulated $^{86}Rb^+$ flux to an extent nearly similar to that of 10 μM nicotine, which has been shown to produce a near-maximal $\alpha4\beta2$ effect in this assay (Marks et al., 1999, 2004). Indeed, the EC_{50} values for these compounds in mouse brain were similar to the values determined with the use of high-sensitivity $\alpha4(u)\beta2(u)$ expressed in *X. laevis* oocytes. Furthermore, thalamic responses to A-162035 and A-168939 were blocked by the $\alpha4\beta2$ antagonist DHβE. These observations support the idea that high-sensitivity $\alpha4\beta2$ represents a native $\alpha4\beta2$ nAChR.

A simple assumption is that the mixed sensitivity $\alpha4\beta2$ responses resulted from expression of different $\alpha4\beta2$ receptors [e.g., $\alpha4_{(3)}\beta2_{(2)}$, and $\alpha4_{(2)}\beta2_{(3)}$] with the high-sensitivity component ($\alpha4_{(2)}\beta3_{(3)}$) corresponding to the receptor expressed from UTR-containing $\alpha4$ and $\beta2$ messages or low $\alpha4/\beta2$ ratios. Biphasic concentration-response curves were fit by the sum of two Hill equations, assuming independent activation of the two components. Most data were consistent with these assumptions. However, some apparent discrepancies were noted. Chlorisondamine was less potent against $\alpha4(u)\beta2(u)$ than $\alpha4\beta2$ stimulated by 2 μM acetylcholine even though responses were expected to be predominantly (≥97%) from the receptor with high-sensitivity to acetylcholine in both measurements. Nicotine was a partial agonist (24%) at $\alpha4(u)\beta2(u)$, yet it seemed to be essentially a full agonist at the high-sensitivity component of ferret mixed sensitivity $\alpha4\beta2$ expressed in oocytes and at the high-sensitivity component in mouse thalamic synaptosomes. The explanation is not known, but it is possible that high- and low-sensitivity components result from differences in the binding sites within the nAChR pentamer (e.g., α-α versus α-β), nonindependent α-β dimer function conditioned by the fifth subunit in the pentamer, or perhaps larger scale interactions in receptor clusters.

The UTR-containing mRNAs that facilitated expression of high-sensitivity $\alpha4\beta2$ and $\alpha3\beta2$ represent naturally ex-

pressed messages. UTRs can regulate expression at the mRNA and/or protein levels. Within some UTRs are sequences that can interact with regulatory proteins, RNA sequences, or other molecules and thereby provide means for regulating the expression of the encoded protein (Morris and Geballe, 2000; Mazumder et al., 2003; Wilusz and Wilusz, 2004). Through such processes, the expression of high- and low-sensitivity nAChR subforms may be regulated in neurons, possibly developmentally, according to cell type, or in response to various extracellular messengers. Such regulatory processes potentially could affect a variety of nAChR physiological and pharmacological actions, including nicotine dependence, antinociception, and cognitive function.

Acknowledgments

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