0022-3565/97/2801-0346$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 280, No. 1

Printed in U.S.A.
JPET 280:346–356, 1997

Pharmacological Characterization of Recombinant Human Neuronal Nicotinic Acetylcholine Receptors hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 Expressed in *Xenopus* Oocytes

LAURA E. CHAVEZ-NORIEGA, JAMES H. CRONA, MARK S. WASHBURN, ARTURO URRUTIA, KATHRYN J. ELLIOTT and EDWIN C. JOHNSON

SIBIA Neurosciences, Inc., La Jolla, California

Accepted for publication September 5, 1996

ABSTRACT

Human neuronal nicotinic acetylcholine receptors (nAChRs) hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 were expressed in *Xenopus* oocytes and tested for their sensitivities to the nicotinic agonists acetylcholine (ACh), nicotine, cytisine (CYT) and 1,1-dimethyl-4-phenylpiperazinium (DMPP) and the nAChR. antagonists mecamylamine (MEC), d-tubocurarine and dihydro-β-erythroidine. CYT was the least efficacious agonist at hnAChRs containing β2 subunits, but it displayed significant activity at hα2β4, hα3β4, hα4β4 and hα7 nAChRs. ACh was one of the most efficacious agonists at all hnAChRs, except at hα3β2, where DMPP was markedly more efficacious than ACh. ACh was among the least potent agonists at all hnAChRs. The rank order of potency displayed by hα3β2 and hα3β4 nAChRs (DMPP≈CYT≈nicotine>ACh and DMPP > CYT≈nicotine>ACh, respectively), differs from that reported for their rat homologs (Luetje and Patrick, 1991; Covernton *et al.*, 1994). The agonist profile observed in hα7 also differs from that reported for its rat homolog (Seguela *et al.*, 1993). Human α4β2 and hα4β4 nAChRs were more sensitive to dihydro-β-erythroidine than d-tubocurarine, whereas hα7 and hα3β4 were more sensitive to d-tubocurarine than dihydro-β-erythroidine. These antagonists were equipotent at hα2β2, hα3β2 and hα2β4 nAChRs. MEC (3 μM) inhibited hα2β4 and hα4β4 nAChRs by > 80%, whereas hα2β2, hα4β2 and hα7 nAChRs were inhibited by approximately 50%. Taken together, the differential sensitivities observed at various recombinant hnAChR subtypes indicate that both α and β subunits contribute to the pharmacology of these ligand-gated channels. The unique selectivity profiles displayed by human nAChRs constitute a valuable tool for the development of selective nicotinic analogs as potential therapeutic drugs.

nAChRs are ligand-gated ion channels activated by the neurotransmitter ACh and are distributed throughout the peripheral and central nervous system (Clarke *et al.*, 1985; Wada *et al.*, 1989; Dineley-Miller and Patrick, 1992; Séguéla *et al.*, 1993; Rubboli *et al.*, 1994). To date, a gene family encoding 11 nAChR subunits has been identified (Elgoyhen *et al.*, 1994; for a review see Sargent, 1993). We and others have cloned nine human nAChR subunits: α2, α3, α4, α5, α6, α7, β2, β3 and β4 (Elliott *et al.*, 1996; Fornasari *et al.*, 1990; Chini *et al.*, 1992; Anand and Lindstrom, 1990; Tarroni *et al.*, 1992; Doucette-Stamm *et al.*, 1993; Peng *et al.*, 1994; Willoughby *et al.*, 1993). The stoichiometry of recombinant nAChRs expressed in *Xenopus* oocytes is thought to be $(\alpha x)_2(\beta y)_3$ (Anand *et al.*, 1991; Cooper *et al.*, 1991); however, in recombinant expression systems α7, as well as α8 and α9 can form functional homooligomeric receptors (Couturier *et al.*, 1990; Gerzanich *et al.*, 1994; Elgoyhen *et al.*, 1994).

Pharmacological and functional studies of recombinant rat and chicken nAChRs expressed in *Xenopus* oocytes have revealed a large diversity among the different subunit combinations (Luetje and Patrick, 1991; Connolly *et al.*, 1992; see Sargent, 1993 and Papke, 1993 for review). Recent reports on the functional characterization of hα7 (Peng *et al.*, 1994; Gopalakrishnan *et al.*, 1995) and hα4β2 nAChRs (Gopalakrishnan *et al.*, 1996) indicate that the human homologs are also pharmacologically and functionally diverse.

Many different subtypes of nAChRs have been reported in a variety of neurons; nAChRs are present in both pre- and postsynaptic structures in the rodent and chick central nervous system (reviewed by Sargent, 1993 and Clarke, 1995).

Received for publication April 12, 1996.

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptors; ACh, acetylcholine; NIC, (-)nicotine; CYT, cytisine; DMPP, 1,1-dimethyl-4-phenylpiperazinium; MEC, mecamylamine; d-Tubo, d-tubocurarine; DHβE, dihydro-β-erythroidine; DRCs, dose-response curves; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid].

One approach to gain insight into the molecular composition of native nAChRs has been to compare their functional and pharmacological profiles with those observed using recombinant receptors. The molecular composition of some native chick and rat nAChRs has been proposed based on their pharmacological profile and the characteristics of their macroscopic currents (Mulle *et al.*, 1991; Alkondon and Albuquerque, 1993; Covernton *et al.*, 1994; Zhang *et al.*, 1994). However, at the single-channel level, a good correlation has not yet been established between native and recombinant nAChRs tested to date (Connolly *et al.*, 1995, for reviews see McGehee and Role, 1995; Sargent, 1993 and Papke, 1993).

Administration of nicotinic agonists to rodents increases locomotor activity and enhances learning and memory, as shown in several behavioral tests (Clarke and Kumar, 1983; Levin *et al.*, 1993). In human neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, there is a significant reduction in nAChR number (Rinne *et al.*, 1991; Nordberg, 1994), and administration of nAChR agonists may ameliorate many of the motor and cognitive deficits associated with these diseases (Baron, 1986; Newhouse *et al.*, 1988) and other motor disorders, such as Tourette's syndrome (Moss *et al.*, 1989). More recently, a missense mutation in the hnAChR subunit α4 was found to be associated with a form of familial frontal lobe epilepsy (Steinlein *et al.*, 1995). Identification and characterization of the hnAChR subtypes involved in these phenomena may therefore be critical in the development of subtype-specific nAChR modulators for therapeutic purposes.

Human nAChR subunits α2, α3, α4, α5, α6, α7, β2, β3 and β4 show 91–99% amino acid identity to their rat homologs in their extracellular amino terminal domain (Anand and Lindstrom, 1990; Fornasari *et al.*, 1990; Chini *et al.*, 1992; Doucette-Stamn *et al.*, 1993; Willoughby *et al.*, 1993; Peng *et al.*, 1994; Elliott *et al.*, 1996). These differences in the deduced amino acid sequences may affect the properties of nAChRs: substitution of a single amino acid residue in the extracellular amino terminal region of α3 (Hussy *et al.*, 1994) and α7 (Galzi *et al.*, 1991) nAChRs subunits has been shown to dramatically affect the pharmacology of recombinant nAChRs. Studying the properties of hnAChRs using heterologous expression may therefore provide valuable insights into the composition, function and pharmacology of native hnAChRs. We now report that, when expressed in *Xenopus* oocytes, recombinant hnAChRs display unique sensitivities to nAChR agonists and antagonists, and the pharmacology of some of these hnAChRs differs from that reported for their rat homologs.

Methods

Clones. The hnAChR subunits α2, α3, α4-2, β2, β4 and α7 were cloned from cDNA libraries prepared from human brain and the human IMR32 neuroblastoma cell line (Elliott *et al.*, 1996). GenBank access numbers for the cDNA nucleotide sequences are U62431-U62439 (α2-α7 and β2-β4, respectively). The 5′ untranslated region of α2, α4-2, β2 and α7 cDNA was removed and replaced with a Kozak consensus ribosomal binding site, 5′-GCCACC-3′ (Kozak, 1987). The cDNAs were subcloned into different expression vectors, as indicated in Elliott *et al.* (1996), except that the KEβ2RBS insert was subcloned into a pCMV vector modified by the insertion of a T7 promoter. *In vitro* transcripts were prepared using MegaScript T7 or SP6 capped RNA transcription kits (Ambion, Inc., Austin, TX).

Xenopus oocyte injection. *Xenopus laevis* frogs were purchased from Nasco (Fort Atkinson, WI) or *Xenopus* One (Beverly Hills, FL). Mature females were anesthetized by immersion in a 0.15 to 0.3% tricaine methanesulfonate solution and oocytes were surgically removed. The follicular cell layer was partially removed after incubation for 2 to 3 hr. in a solution containing (in mM): NaCl (100), KCl (2), $MgCl_2$ (1), HEPES (5) and 1.5 mg/ml collagenase A. Defolliculation was completed manually in most cases. Oocytes were injected with 10 to 50 nl containing 10 to 100 ng of combinations of hnAChRs subunits $\alpha_X + \beta_X$ *in vitro* synthesized RNA. Pair-wise subunit combinations were injected at a 1:1 ratio. After injection, oocytes were incubated at 19°C for 3 to 5 days in a solution containing (in mM). NaCl (77.5), KCl (2), $CaCl_2$ (1.8), $MgCl_2$ (1), HEPES (5), Na-Pyruvate (5), with penicillin/streptomycin (10 ml/liter).

Drugs. ACh, NIC, CYT, DMPP, d-Tubo, MEC, atropine and collagenase A were purchased from Sigma Chemical Co. (St. Louis, MO). DHβE was purchased from Research Biochemicals International (RBI, Natick, MA). Stock solutions of agonists and antagonists were prepared and frozen. Individual aliquots were thawed and diluted in standard Ringer at the desired final concentrations.

Recording procedures. Oocytes were examined for functional expression 2 to 5 days after RNA injection using a two-electrode voltage-clamp protocol, with a GeneClamp 500 (Axon Instruments, Foster City, CA), or an Oocyte Clamp OC-725B (Warner Instrument Corp., Hamden, CT) amplifier. Axotape and pCLAMP software (Axon Instruments), Origin (Microcal, Northampton, MA) and Prizm (Graphpad, San Diego, CA) software were used for data acquisition and analysis. Membrane potential was held at either -80 mV (for partial agonist DRCs and for antagonist experiments) or -60 mV (for full agonist DRCs); experiments were performed at room temperature (19–23°C). Microelectrodes were filled with a 3 M KCl solution (2–4 MΩ resistance). The extracellular recording solution (standard Ringer's) contained (in mM): NaCl (115), KCl (2.5), $CaCl_2$ (1.8), HEPES (10), atropine (0.001), pH 7.3. Perfusion solutions were gravity fed into the recording chamber (Warner Instruments, capacity: 110 μl) at a rate of 10 to 13 ml/min and were extruded at the opposite side of the chamber by vacuum; perfusate exchange was performed manually by switching between solenoid valves/reservoirs. Under these conditions, saturating concentrations of agonists could routinely activate currents with 0 to 100% rise-times of $<$200 msec, *e.g.*, response in hα7 in figure 1. Agonists were applied for approximately 10 sec in most experiments, although shorter ($\approx$5 sec) or longer ($\approx$20 sec) applications were also tested. Peak response amplitudes were measured and used in the determination of agonist and antagonist properties. Oocytes were washed in drug-free Ringer's for 3 to 10 min between successive drug applications for agonist and antagonist studies, except where otherwise indicated.

Agonist DRCs were obtained using two different methods. 1) For partial DRCs, responses were normalized to 1 μM ACh in all hnAChR subunit combinations, except hα7, normalized to 10 μM ACh. The normalizing dose of ACh was applied several times to each oocyte during the course of an experiment to check for desensitization; data were rejected if responses to the normalizing dose fell below 80% of the original response. 2) For full agonist DRCs, responses from each oocyte were normalized to the maximal response for each agonist tested and used to generate EC_{50} and n_H estimates. For comparison of relative agonist efficacies, the agonist responses for each oocyte were normalized to the response elicited by an EC_{80} dose of ACh (EC_{20} for hα3β2). For full DRCs, hnAChR subunit combinations containing β4 subunits (hα2β4, hα3β4 and hα4β4) and hα7 were tested in a Ringers solution containing 0.18 mM [Ca^{2+}], to reduce the contribution of Ca^{2+}-activated Cl^- currents (Miledi and Parker, 1984) in agonist-induced responses. In this low [Ca^{2+}] Ringers solution, β2-containing hnAChRs showed very small agonist-induced currents ($\leq$40 nA to 1–3 mM ACh), possibly related to their sensitivity to external Ca^{2+} (Mulle *et al.*, 1992; Vernino *et al.*, 1992; Mahaffy *et al.*, 1996), and therefore were tested in standard Ringer's (1.8 mM [Ca^{2+}]).

The sensitivity to the nAChR antagonists d-Tubo, DHβE and MEC was tested in standard Ringer's solution. Each oocyte was tested at all concentrations indicated for the d-Tubo and DHβE experiments, except for d-Tubo on hα2β2 and hα3β4 nAChRs. For the latter, a different group of oocytes was tested with each antagonist dose, and one curve was fitted to the (averaged) data points. The activity of MEC was assessed at one dose (3 μM), due to the incomplete reversibility of the block by this antagonist.

Data analysis. Dose-response curves for agonists (full DRCs) and antagonists (d-Tubo and DHβE) were fitted by nonlinear regression to the equations: $I = I_{max}/[1 + (EC_{50}/Ag)^n]$ or $I = I_{max} - I_{max}/[1 + (IC_{50}/An)^n]$ wherein I_{max} = maximal normalized current response (in the absence of antagonist for inhibitory curves), Ag = agonist concentration, An = antagonist concentration, EC_{50} = agonist concentration eliciting half maximal current, IC_{50} = antagonist concentration eliciting half maximal current, and n = Hill coefficient. Antagonist curves were constrained to I_{max}=1 and I_{min}= 0. For agonist efficacy curves, I_{min} was constrained to 0, but I_{max} was not constrained.

Concentration data (EC_{50} and IC_{50} estimates) are shown as the geometric means ± S.D. Hill coefficient and efficacy estimates are shown as the arithmetic mean ± S.D. For the antagonists, IC_{50} values were converted to K_b values using the Leff-Dougall (Leff and Dougall, 1993) variant of the Cheng-Prusoff equation: $K_b = IC_{50}/((2 + ([Ag]/[A_{50}])^n)^{1/n} - 1)$, where Ag is the agonist, A_{50} is the EC_{50} value for the agonist and n = Hill coefficient.

Statistical tests. The geometric values for the EC_{50} or K_b data were tested for significant differences between receptor subtypes using a one-way analysis of variance followed by a Student-Newman-Keuls or Dunn's test for pairwise multiple comparisons. The Student-Newman-Keuls and the Dunn's tests (SigmaStat, ver. 1.01, Jandel Corporation, San Rafael, CA) provide a significance level of $P < .05$, but do not provide the absolute P value; therefore the differences may be of greater significance than stated in the text and tables. Differences in arithmetic n_H values for a given agonist between a β4- and a β2-containing hnAChRs, or differences in geometric IC_{50} values between DHβE and d-Tubo for each subunit combination were tested for significance with an unpaired two-tailed t test. The significance of differences in agonist potency from partial DRCs (or the potency of MEC) among hnAChRs subtypes, was tested with the Kruskal-Wallis one-way analysis of variance; followed by pairwise multiple comparisons with the Dunn's test (SigmaStat, ver. 1.01, Jandel Corporation).

Results

Human recombinant nAChRs display differential sensitivities to nicotinic agonists. The nicotinic agonists

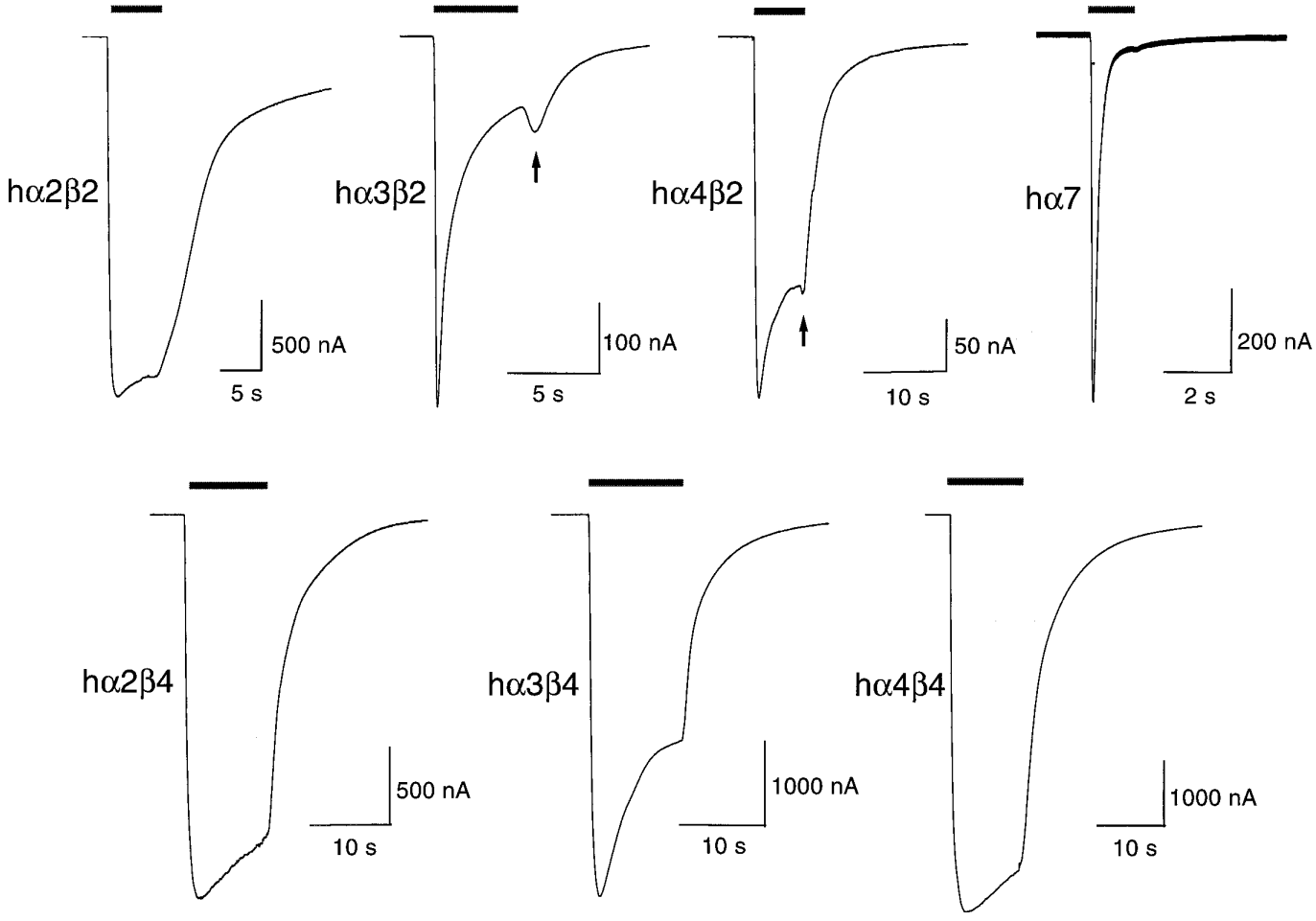

Fig. 1. Representative traces showing the current responses to maximally effective concentrations of ACh in oocytes injected with mRNA encoding various human nicotinic receptors. Data shown in figures 1 to 3 were obtained from oocytes voltage-clamped at -60 mV. Of the β2-containing receptors, hα3β2 receptors showed the fastest decay kinetics to ACh application. Similarly, hα3β4 receptors showed more apparent desensitization than did hα2β4 or hα4β4 receptors (bottom row). Currents recorded from hα7 nAChRs decayed very rapidly (upper right panel). Note the transient inward current observed in hα3β2- and hα4β2-injected oocytes upon removal of agonist (arrows). Maximally effective concentrations of ACh for the oocytes shown here were 300 μM for hα2β4 and hα4β4 receptors, 1 mM for hα2β2, hα3β4, hα4β2 and hα7 receptors and 3 mM for hα3β2 receptors

ACh, NIC, CYT and DMPP produced dose-dependent inward currents in voltage-clamped oocytes expressing different hnAChRs subunit combinations. The kinetics of agonist-induced currents were found to differ among the various subunit combinations (figs. 1 and 2A). Of the heteromeric nAChRs, currents generally decayed most rapidly in hα3β2 nAChRs; in contrast, currents elicited in hα2β2, hα2β4 and hα4β4 showed relatively little desensitization in the continued presence of high concentrations of agonists (fig. 1). Currents recorded from hα3β4 decayed substantially faster than those recorded in hα2β4 or hα4β4 nAChRs (figs. 1 and 2A). Responses from hα7 nAChRs decayed much more rapidly than those from any of the heteromeric nAChR subunit combinations (fig. 1). Agonist-dependent differences in the decay rate were also observed (for example in hα3β2: fig. 2, left panels), where a markedly faster decay rate was observed with DMPP than with CYT.

Human nAChRs subunit combinations exhibited distinct sensitivities to nicotinic agonists. Full dose response curves obtained for ACh, NIC, DMPP and CYT are shown in figure 3; data are summarized in table 1. The rank order of potency (EC_{50} estimates) derived from the full dose-response curves was the following (>indicates the significance level is $P < .05$ or higher, see "Methods"): hα2β2: DMPP≈NIC≈CYT≈ACh, DMPP > ACh; hα2β4: NIC≈DMPP > CYT>ACh; hα3β2: DMPP≈CYT≈NIC>ACh; hα3β4: DMPP > CYT≈NIC>ACh; hα4β2: CYT>NIC>DMPP > ACh; hα4β4: CYT>NIC> DMPP≈ACh and hα7: DMPP > CYT≈NIC≈ACh. These results show that ACh is the least potent of all agonists tested in most subunit combinations (hα2β4, hα3β2, hα3β4 and hα4β2).

Steeper agonist dose-response curves (fig. 3) and thus higher Hill coefficient values (table 1) were apparent in β4-containing hnAChRs, compared to β2-containing receptors coexpressed with the same α subunit. The differences in the Hill coefficients between hα2β2 and hα2β4 nAChRs for ACh, NIC and DMPP were significant ($P < .05$). Hill coefficient values were significantly larger for ACh, DMPP and CYT in hα3β4, compared to hα3β2 nAChRs ($P < .05$). Hill coefficient estimates from hα4β4 nAChRs were also significantly larger than those from hα4β2 for ACh, DMPP and CYT ($P < .05$). The large Hill coefficient for CYT on hα2β2 nAChRs is likely due to the low efficacy of the agonist on this subtype, which gives the smallest maximal responses.

Marked subtype-specific differences were also apparent in the relative efficacies displayed by these different nAChR agonists. CYT was least efficacious at β2-containing hnAChRs (hα2β2, hα3β2 and hα4β2), in contrast to its efficacy shown on β4-containing hnAChRs, hα2β4, hα3β4 and hα4β4. CYT displayed full agonist activity only at ha7 (fig. 3; table 1). ACh was the most or among the most efficacious agonists at all hnAChR subunit combinations except on hα3β2 hnAChRs, where DMPP was markedly more efficacious than ACh.

A dose-dependent increase in the rate of decay of agonist-induced inward currents was observed in all subunit combinations; this appeared more pronounced in hα3β2, hα3β4 (fig. 2) and hα7 nAChRs. This increase in the apparent rate of desensitization was also accompanied by a "rebound" inward current upon the removal of high doses of some agonists in some hnAChRs (figs. 1 and 2, arrows). This rebound current has been shown to be an indication of agonist-dependent open-channel block in native (Maconochie and Knight, 1992) and recombinant (Bertrand *et al*, 1992a) neuronal nAChRs. Long-lasting nAChR desensitization is supported by the observation that in some oocytes where an EC_{80} or EC_{20} dose of ACh was tested both before and up to 15 min after the completion of a full agonist DRC, the current amplitude to the second ACh application was reduced. This was more evident on hα3β2-expressing nAChRs, but was also observed in β4-containing hnAChRs. This long-lasting form of desensitization was not observed in oocytes expressing hα7 nAChRs.

These observations indicate that application of mid to high

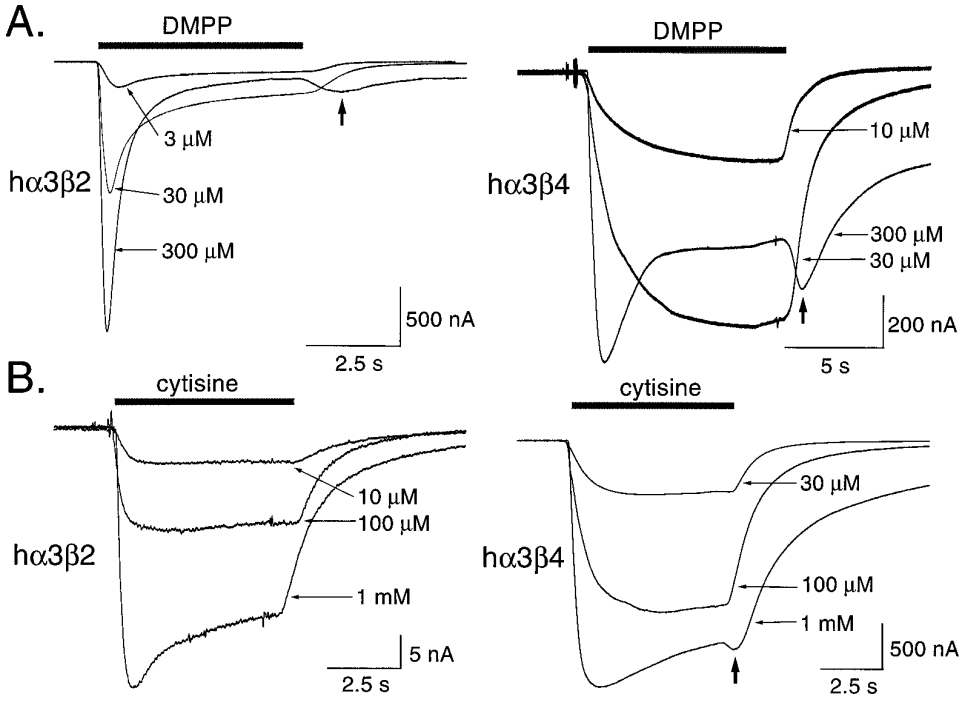

Fig. 2. Dose-dependent responses elicited by the application of the nicotinic agonists DMPP and CYT in oocytes expressing the hnAChRs subunit combinations hα3β2 or hα3β4. A, DMPP produced rapidly decaying responses in an oocyte injected with hα3β2 mRNA (left) but more slowly decaying responses in an hα3β4-injected oocyte (right). In each of these oocytes, application of a high agonist concentration produced a rapidly decaying response followed by a transient inward current when switching from DMPP-containing to control medium (arrows). B, Current responses elicited by the application of various concentrations of CYT showed similar decay properties in oocytes expressing hα3β2 or hα3β4 nAChRs. As with DMPP, switching from cytisine-containing to control medium produced a small inward current not seen at lower agonist concentrations. A through D represent data obtained from four different oocytes.

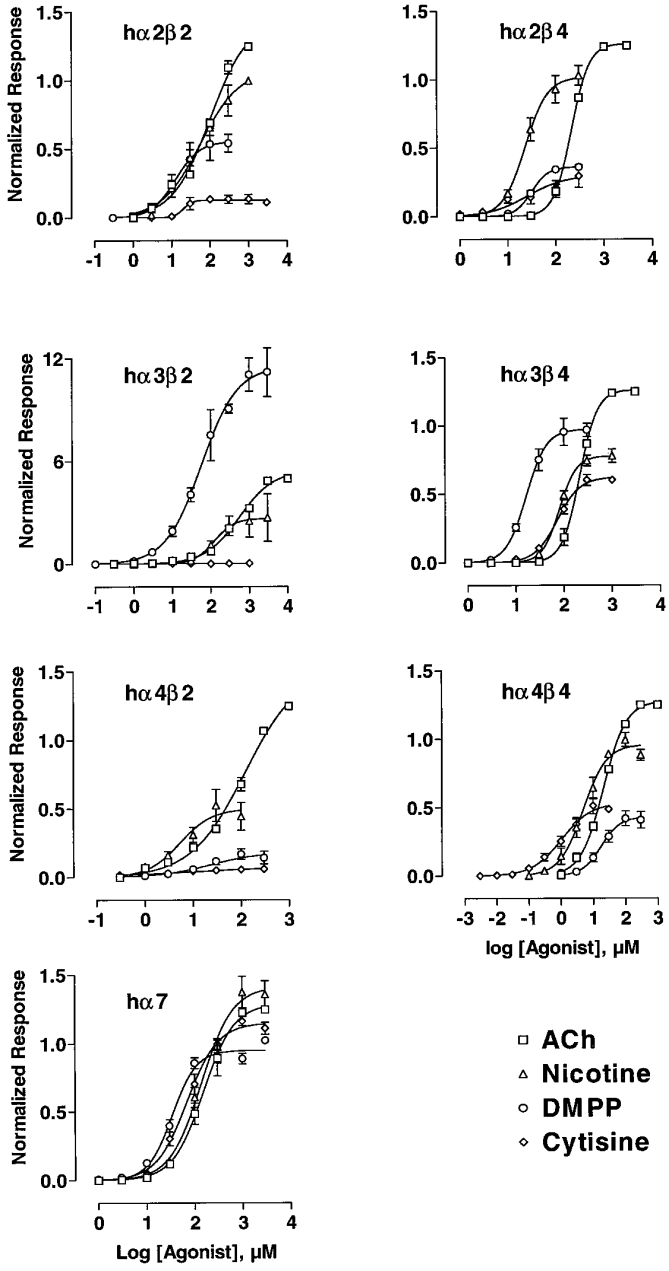

Fig. 3. Full dose-response curves for ACh, (-) NIC, DMPP and CYT on recombinant hnAChRs. Current responses in each oocyte were normalized to the EC_{80} (or EC_{20} for hα3β2) ACh response recorded in the same oocyte. Data points indicate the mean ± S.E.M. of three to six oocytes. Where no error bars are seen, they are smaller than the symbols.

agonist concentrations, such as those required to achieve saturation of agonist DRCs, can result in desensitization and/or agonist-induced channel block of neuronal nAChRs. Both can contaminate efficacy, potency and Hill coefficient values; therefore, these estimates may not directly reflect the interaction of the ligand with the nAChRs.

To address this issue, we have compared the rank order of potencies estimated from full dose-response curves with those obtained in a separate series of experiments from partial dose-response curves (fig. 4), similar to those reported for recombinant rat nAChRs (Luetje and Patrick, 1991; Connolly *et al.*, 1992; Covernton *et al.*, 1994). These experiments were designed to test the relative sensitivity of hnAChRs at agonist concentration ranges where desensitization would be expected to be small (0.3 to 10–30 μM). They also serve as a comparison to the only other study that has compared the agonist profiles among all six pair-wise nAChRs subunit combinations using the rat homologs (Luetje and Patrick, 1991). Data were rejected if responses to the normalizing dose fell below 80% of the initial response (see "Methods"). We found that the relative potency displayed by these four agonists, in the ranges $\leq$ 30 μM, was similar using both methods for all hnAChRs except hα2β4. In hα2β4 the relative potency of DMPP, NIC and ACh appeared different: NIC≈ACh>DMPP with partial DRCs (fig. 4), whereas NIC>DMPP > ACh was observed at 10–30 μM in the full DRCs (fig. 3). Using either method, CYT elicited the largest responses at doses $\leq$3 μM in this subunit combination. From the partial DRCs, it is apparent that CYT is the least potent agonist at β2-containing hnAChRs, whereas it is the most potent agonist at hα2β4 and hα4β4 nAChRs. These results are similar to what has been reported for their rat homologs and are consistent with the idea that β subunits also contribute to the pharmacology of neuronal nAChRs (Luetje and Patrick, 1991).

To analyze the effect of agonist-induced nAChR desensitization and/or channel block on the Hill coefficient estimates obtained from the fits to the full DRCs, we examined the slope of log-log plots from the partial agonist DRCs. The slope of log-log plots of DRCs at low agonist concentration ranges approximates the Hill coefficient (cf. Connolly *et al.*, 1992; Covernton *et al.*, 1994; Cohen *et al.*, 1995). Using low concentrations of ACh ($\leq$30 μM) to minimize nAChR desensitization and the contribution of the endogenous Ca^{2+}-activated Cl^- current, we have compared the slopes of these dose-response log-log plots among the different hnAChR subtypes. The slopes of the ACh log-log plots were markedly steeper for β4-containing hnAChRs (and hα7) than for β2-containing hnAChRs (fig. 5). Log-log plots obtained for the other agonists also displayed shallower slopes for nAChRs containing the β2 subunit than those containing β4 subunits (data not shown). The differences observed in log-log DRC slopes between β2- and β4-containing hnAChRs are in agreement with the results obtained with the n_H estimates derived from the full DRCs.

Recombinant hnAChRs show a unique sensitivity to nAChRs blockers. We have tested the sensitivity of these recombinant hnAChRs to the nAChR antagonists d-Tubo, DHβE and MEC. Dose-response curves for d-Tubo and DHβE inhibition were constructed for each hnAChR subunit combination; sensitivity to MEC was tested at a single concentration (3 μM; see "Methods"). The agonist and dose to test these antagonists on each subunit combination were selected on the basis of 1) potency: the most or one of the most potent agonist was used and, 2) magnitude of the response: a concentration eliciting a large response, but relatively small desensitization upon repeated application (see fig. 4). The agonists and doses selected were the following: hα2β2: 10 μM NIC; hα2β4: 30 μM ACh; hα3β2: 10 μM DMPP; hα3β4: 10 μM DMPP; hα4β2: 10 μM ACh; hα4β4: 10 μM NIC; hα7: 100 μM ACh.

DHβE and d-Tubo reversibly inhibited agonist-induced currents in oocytes expressing these different hnAChRs (fig. 6). The reversibility of nicotinic responses after MEC appli-

TABLE 1
Comparison of potency and efficacy of nAChRs agonists on recombinant hnAChRs[a]

Agonist	h$\alpha2\beta2$	h$\alpha2\beta4$	h$\alpha3\beta2$	h$\alpha3\beta4$	h$\alpha4\beta2$	h$\alpha4\beta4$	h$\alpha7$
ACh (*N*)	(4)	(4)	(5)	(4)	(4)	(4)	(5)
EC_{50}	68.67 μM	82.57 μM	442.90 μM	203.14 μM	68.05 μM	19.68 μM	179.57 μM
(−S.D., +S.D.)	(61.56, 76.61)	(71.61, 95.21)	(298.78, 656.55)	(191.15, 215.88)	(54.15, 85.51)	(16.45, 23.53)	(132.06, 244.17)
n_H	1.22 ± 0.1	1.70 ± 0.1	1.17 ± 0.1	2.24 ± 0.2	1.02 ± 0.04	1.28 ± 0.04	1.46 ± 0.02
I_{max}	1.25	1.25	5.0	1.25	1.25	1.25	1.25
NIC (*N*)	(6)	(4)	(6)	(4)	(5)	(4)	(4)
EC_{50}	19.23 μM	20.71 μM	132.44 μM	80.30 μM	5.47 μM	5.02 μM	113.34 μM
(−S.D., +S.D.)	(15.64, 23.65)	(19.34, 22.19)	(67.23, 260.89)	(78.20, 82.47)	(4.48, 6.68)	(2.53, 9.97)	(65.84, 195.11)
n_H	1.38 ± 0.1	2.24 ± 0.1	2.54 ± 0.7	2.38 ± 0.1	1.26 ± 0.05	1.28 ± 0.1	1.61 ± 0.1
I_{max}	0.77 ± 0.11	1.03 ± 0.07	2.84 ± 0.52	0.78 ± 0.05	0.52 ± 0.12	1.0 ± 0.05	1.38 ± 0.11
DMPP (*N*)	(4)	(4)	(3)	(4)	(4)	(4)	(4)
EC_{50}	11.18 μM	22.77 μM	55.87 μM	18.67 μM	17.99 μM	18.71 μM	30.86 μM
(−S.D., +S.D.)	(8.48, 14.74)	(18.47, 28.06)	(44.02, 70.93)	(15.60, 22.36)	(17.15, 18.86)	(11.70, 29.93)	(3.15, 41.14)
n_H	1.44 ± 0.1	2.16 ± 0.2	0.97 ± 0.1	1.93 ± 0.3	1.24 ± 0.05	1.56 ± 0.1	2.18 ± 0.4
I_{max}	0.53 ± 0.02	0.36 ± 0.02	11.20 ± 1.42	0.98 ± 0.07	0.14 ± 0.05	0.42 ± 0.05	1.02 ± 0.01
CYT (*N*)	(4)	(4)	(3)	(4)	(5)	(4)	(4)
EC_{50}	25.43 μM	38.86 μM	67.09 μM	72.18 μM	2.61 μM	0.90 μM	71.42 μM
(−S.D., +S.D.)	(13.78, 46.92)	(31.15, 48.47)	(51.12, 88.04)	(64.77, 80.44)	(1.69, 4.05)	(0.84, 0.97)	(45.56, 111.96)
n_H	13.37 ± 5.04	1.01 ± 0.01	0.95 ± 0.04	1.91 ± 0.1	0.61 ± 0.06	1.0 ± 0.04	1.41 ± 0.03
I_{max}	0.13 ± 0.02	0.29 ± 0.08	0.038 ± 0.003	0.60 ± 0.04	0.06 ± 0.01	0.51 ± 0.06	1.16 ± 0.03

[a] Potency (EC_{50}) expressed as geometric mean, Hill coefficient (n_H) and efficacy (I_{max}) are expressed as the arithmetic mean ± S.E.M. *N* indicates the number of oocytes included in the estimates and I_{max} represents the fraction of the ACh EC_{80} response (EC_{20} for h$\alpha3\beta2$).

cation (3 μM) was variable. In some cells, full recovery was not observed after prolonged (10–15 min) washout in drug-free Ringer's. A differential sensitivity to the three antagonists was observed (figs. 7–9). Table 2 summarizes the K_b estimates obtained from the Leff-Dougall variant of the Cheng-Prusoff equation (Leff and Dougall, 1993), which corrects for both the potency of the agonist used and its Hill coefficient from the agonist DRCs. The K_b estimates for DHβE and d-Tubo from the DRCs (fig. 7) indicate that h$\alpha4\beta2$ and h$\alpha4\beta4$ nAChRs are more sensitive to block by DHβE than d-Tubo ($P < .01$, *t* test), whereas h$\alpha7$ ($P < .01$, *t* test) and h$\alpha3\beta4$ are more sensitive to block by d-Tubo than DHβE. In contrast, no significant difference in the K_b estimates for these two antagonists was found in h$\alpha2\beta2$, h$\alpha2\beta4$ and h$\alpha3\beta2$ nAChRs ($P > .05$). Human $\alpha4\beta4$ was the nAChR subtype most sensitive to block by DHβE and d-Tubo. K_b values for d-Tubo were significantly lower for h$\alpha4\beta4$ than those of h$\alpha4\beta2$, h$\alpha2\beta4$ and h$\alpha7$ ($P < .05$). The rank order of potency of DHβE was h$\alpha4\beta4$>h$\alpha4\beta2$>h$\alpha2\beta2\approx$h$\alpha3\beta2\approx$h$\alpha2\beta4$>h$\alpha3\beta4\approx$h$\alpha7$ (> indicates the significance level is $P < .05$).

The effect of d-Tubo appeared unusual on some hnAChRs. The inhibition by this antagonist on h$\alpha2\beta4$ nAChRs was more dramatic at later times after the activation of the inward current than at the initial peak (fig. 8). This effect, observed in all six cells tested, was noticeable at concentrations of d-Tubo of 0.3 μM and above. The effect on the kinetics of agonist-induced responses produced by d-Tubo is similar to that produced by MEC, but different from the effect of DHβE on this and other hnAChR subunit combinations tested. Our observations suggest that d-Tubo may act noncompetitively at h$\alpha2\beta4$ nAChRs, in addition to its putative action at the ligand binding site. d-Tubo also appeared to alter the kinetics of agonist-induced responses on h$\alpha4\beta4$, but not h$\alpha2\beta2$ nAChRs (data not shown).

MEC (3 μM) inhibited agonist-induced responses by >80% in h$\alpha2\beta4$ and h$\alpha4\beta4$ and by ≈50% in h$\alpha2\beta2$, h$\alpha4\beta2$ and h$\alpha7$ nAChRs (fig. 9). The sensitivity to MEC observed in h$\alpha4\beta4$ nAChRs was significantly more than that observed in h$\alpha2\beta2$, h$\alpha4\beta2$ or h$\alpha7$ hnAChRs ($P < .05$ Dunn's test).

Discussion

We have shown that recombinant hnAChRs display differential sensitivities to nicotinic agonists and antagonists, and that both α and β subunits contribute to the pharmacology of these ligand-gated channels.

Agonist selectivity of recombinant hnAChRs. Full agonist DRCs were obtained for ACh, NIC, DMPP and CYT. Our results indicate that when high agonist concentrations are used, such as those required to reach saturation in DRCs, receptor activation can overlap with agonist-induced receptor desensitization and/or channel block, which can contaminate efficacy, slope and potency estimates. These phenomena are not unique to nAChRs (Luetje and Patrick, 1991; Connolly *et al.*, 1992; Maconochie and Knight, 1992), but are also observed in other ligand-gated channels (for review, see Jones and Westbrook, 1996). Results derived from full DRCs were therefore compared with those obtained from partial DRCs. To our knowledge, this is the first study in which the agonist pharmacology of recombinant nAChRs is evaluated with both partial DRCs (in which agonist concentrations tested are low to minimize receptor desensitization) and full DRCs. At low agonist concentrations, no differences in relative agonist potency were noted between full and partial DRCs in any of the hnAChRs, except for h$\alpha2\beta4$. Also, the relatively larger n_H estimates observed in $\beta4$- compared to their related $\beta2$-containing hnAChRs were observed both in partial DRCs and full DRCs.

An interesting observation is the evaluation of the differential activity of CYT on $\beta2$ *vs.* $\beta4$-containing hnAChRs. Fully saturating DRCs were obtained for CYT in all of the $\beta2$-containing receptors, albeit with very low efficacy, yielding actual EC_{50}, n_H and I_{max} values (see table 1 and fig. 2). The EC_{50}s from these determinations showed similar or

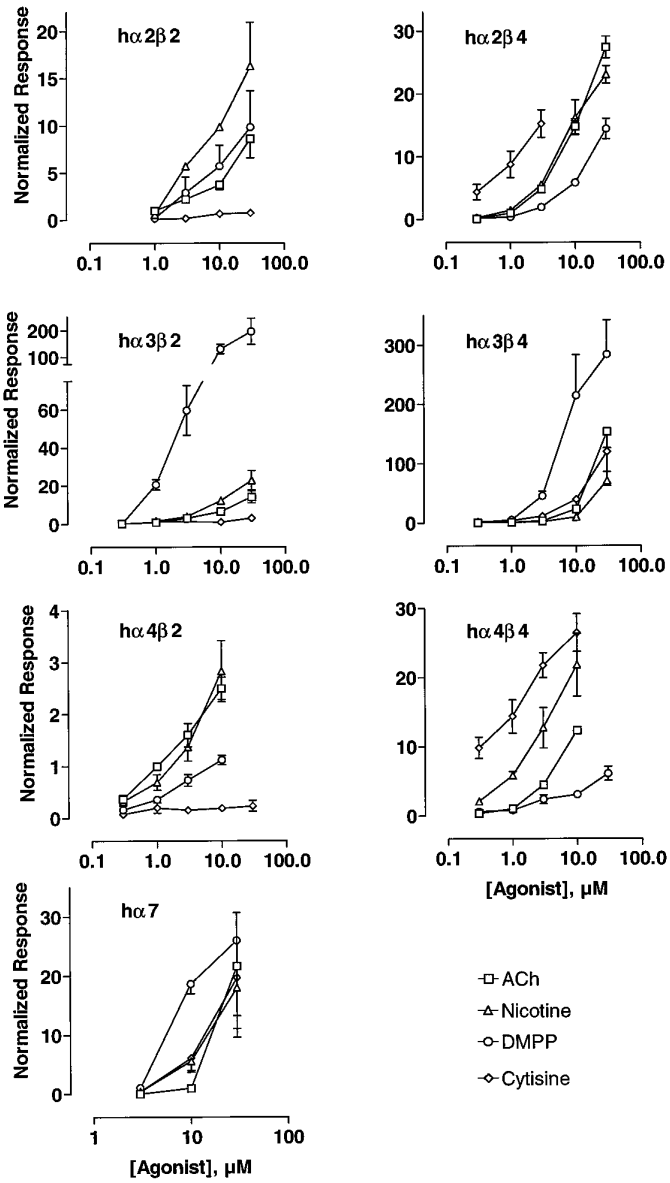

Fig. 4. Partial dose-response curves for hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4 and hα7 nAChRs. Responses to the agonists ACh, (-) NIC, DMPP and CYT, were normalized to the amplitude of the response elicited by 1 μM ACh in the same oocyte, except for hα7, where responses were normalized to 10 μM ACh (response amplitude elicited by 1 or 10 μM ACh = 1). Each symbol represents the mean ± S.E.M. of the responses observed in 3 to 12 oocytes. Where no error bars are seen, they are smaller than the symbols.

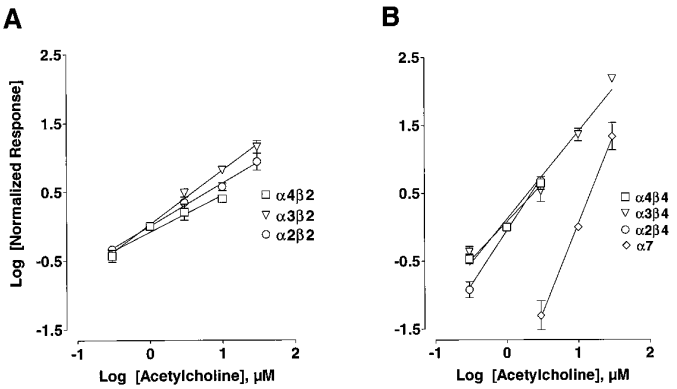

Fig. 5. Log-log plot of the ACh dose-response relation for (A) β2-containing hnAChRs and (B) β4-containing and hα7 hnAChRs. Data points represent the mean ± S.E.M. of the responses normalized to the current elicited by 1 μM ACh in each oocyte (n = 3–10 oocytes). Regression lines were fitted using least squares approximation to the data points. The slope values of these lines are 0.62 for hα2β2, 0.79 for hα3β2, 0.53 for hα4β2, 1.61 for hα2β4, 1.26 for hα3β4, 1.11 for hα4β4 and 2.08 for hα7. Where no error bars are seen, they are smaller than the symbols.

higher potency than the other agonists examined on β2-containing receptors. However, when partial DRCs were constructed, CYT was seen to have a very low potency relative to other agonists at equivalent concentrations (fig. 4). This latter observation is similar to that reported for the rat homologs (Luetje and Patrick, 1991; Covernton *et al.*, 1994) and can be overlooked when the sensitivity to agonists is evaluated from full DRCs.

A marked difference was observed in the kinetics of currents elicited in hα3β4 and hα3β2 nAChRs, with currents elicited on hα3β2 nAChRs decaying more rapidly than those recorded in hα3β4 nAChRs. This is in agreement with the kinetics reported for responses to epibatidine on these hnAChRs (Gerzanich *et al.*, 1995) and for agonist-induced currents in rat α3β2 and α3β4 (Cachelin and Jaggi, 1991; Cohen *et al.*, 1995). The fast kinetics of agonist-induced currents observed in hα7 nAChRs are not different from those reported by Peng *et al.* (1994) and Gopalakrishnan *et al.* (1995) for hα7 and for rat (Séguéla *et al.*, 1993) and chick α7 (Couturier *et al.*, 1990).

The agonist selectivity profile of hα3β2, hα3β4 and hα7 nAChRs reported differs from that reported for their rat homologs. DMPP is more potent than ACh in hα3β2 nAChRs (figs. 3 and 4), whereas the rank order of potency reported for rat α3β2 is DMPP=ACh>NIC>CYT (Luetje and Patrick, 1991). However, two groups have reported that DMPP > ACh for rat α3β2 nAChRs expressed in *Xenopus* oocytes (Cachelin and Jaggi, 1991; Covernton *et al.*, 1994); the reason for this discrepancy is unclear. NIC is more potent than ACh at hα3β2 nAChRs (table 1), in agreement with the recently reported rank order of potency of epibatidine>NIC>ACh for hα3β2 nAChRs expressed in *Xenopus* oocytes (Gerzanich *et al.*, 1995), but different from the profile reported for the rat α3β2 (ACh>NIC: Luetje and Patrick, 1991; Covernton *et al.*, 1994). Although the rank order of potencies for ACh and NIC agree between our work and that of Gerzanich *et al.* (1995), the EC_{50} estimates obtained for ACh and NIC do not. Higher values were observed in this study, compared to those of Gerzanich *et al.* (1995). However, Gerzanich *et al.* used the pSP64T vector for expression of hα3β2 and hα3β4. We examined the potency of ACh and NIC with hβ2 cDNA subcloned into the pSP64T vector and observed ACh and NIC EC_{50}s of 1.75 ± 0.1 μM (n=3) and 0.79 ± 0.22 μM (n=3) for hα2β2, 27.4 ± 8.1 μM (n=4) and 21.1 ± 3.4 μM (n=4) for hα3β2 and 1.3 ± 0.1 μM (n=3) and 0.3 ± 0.1 μM (n=3) for hα4β2. The values that we observed for hα3β2 using the pSP64T vector are similar to those observed by Gerzanich *et al.* for hα3β2. The reason for the differences with these vectors is not understood. By contrast, we did not observe potency differences for ACh or NIC with hα3β4 using hβ4 cDNA (KEβ4.6) subcloned into the pCMV-T7 vector (table 1) compared to the results observed by Gerzanich *et al.* using the pSP64T vector.

DMPP is the most potent agonist at hα3β4 nAChRs (figs. 3

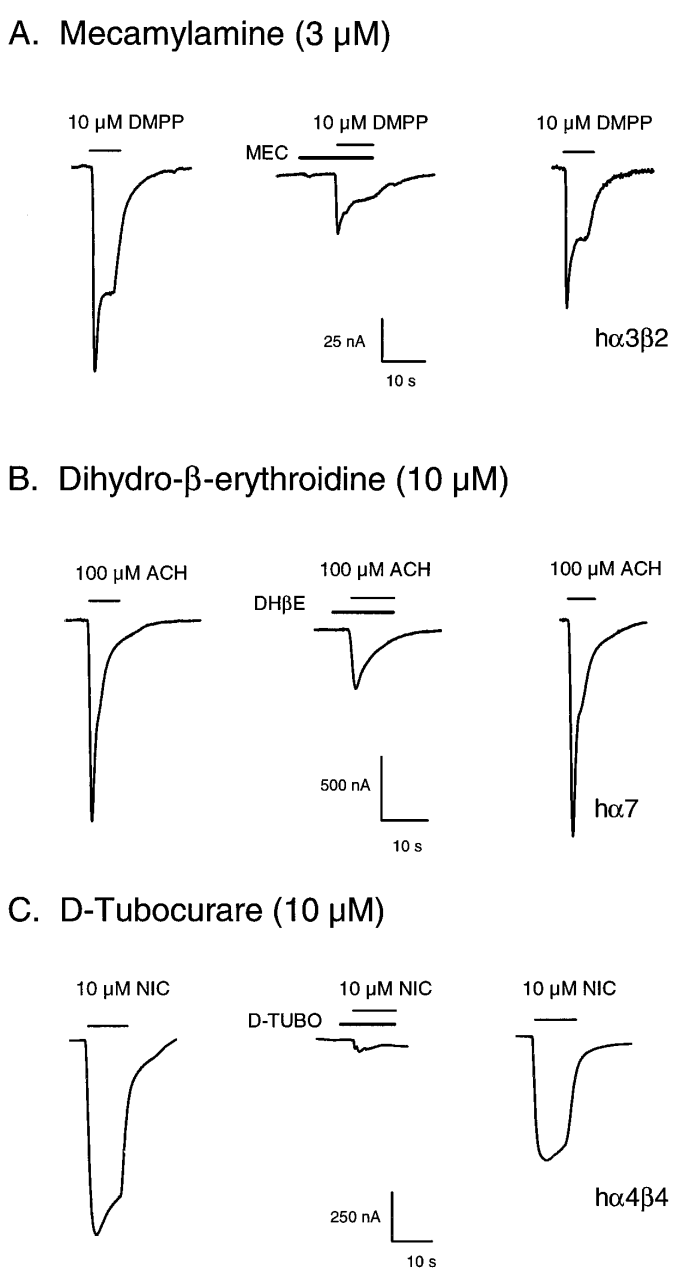

Fig. 6. Inhibition of agonist-induced currents by nicotinic receptor antagonists. Current responses recorded from oocytes expressing h$\alpha3\beta2$ (A), h$\alpha7$ (B) or h$\alpha4\beta4$ (C) nAChRs. Traces shown on each row are from the same oocyte. The time between each application (control, agonist + antagonist and wash) was 5 to 10 min.

and 4). In contrast, the rank order of potency reported for rat $\alpha3\beta4$ is CYT>NIC=ACh≥DMPP (Luetje and Patrick, 1991; Covernton *et al.*, 1994). NIC and ACh are also equipotent in rat $\alpha3\beta4$ nAChRs transiently expressed in mammalian HEK-293 cells (Wong *et al.*, 1995), whereas NIC>ACh at h$\alpha3\beta4$ nAChRs (Table 1), in agreement with the rank order of potency of epibatidine>NIC>ACh reported for h$\alpha3\beta4$ (Gerzanich *et al.*, 1995). The relative efficacies reported for ACh, NIC, CYT and DMPP for rat $\alpha3\beta4$ also differ from the efficacies found in this study (Wong *et al.*, 1995). Taken together, these data indicate that the pharmacology of h$\alpha3\beta4$ and h$\alpha3\beta2$ nAChRs differs from that of their rat homologs.

The agonist sensitivity observed in h$\alpha7$ nAChRs is in agreement with that reported for h$\alpha7$ expressed in *Xenopus* oocytes by Peng *et al.* (1994), but it differs from the sensitivity reported for the rat (NIC>CYT>DMPP > ACh) (Séguéla *et al.*, 1993) and the chick homologs (NIC≈CYT> ACh>DMPP) (Bertrand *et al.*, 1992b) in that DMPP is the most potent agonist at h$\alpha7$ nAChRs. However, Gopalakrishnan *et al.* (1995) recently reported an agonist pharmacology for h$\alpha7$ stably transfected in HEK-293 cells that is closer to that reported for the rat, wherein NIC is the most potent agonist. The reason for this discrepancy is not clear; the full cDNA sequence of Gopalakrishnan *et al.* (1995) for the h$\alpha7$ clones used has not been published.

The rank order of potency observed for nAChR subunit combinations h$\alpha2\beta2$, h$\alpha4\beta2$ and h$\alpha4\beta4$ is similar to that reported for their rat homologs (fig. 4) (Luetje and Patrick, 1991; Connolly *et al.*, 1992). Furthermore, the relative sensitivity to nicotinic agonists recently reported using a $^{86}Rb^{+}$ efflux assay in h$\alpha4\beta2$ nAChRs stably expressed in HEK293 cells (Gopalakrishnan *et al.*, 1996) is in agreement with our results.

Interestingly, even the minor divergence found in the sequence of the amino terminal extracellular domain of α subunits between human and rat may contribute to the pharmacological differences observed between some recombinant hnAChRs and their rat homologs, because a single amino acid substitution in this region can profoundly affect the pharmacology of recombinant nAChRs (Hussy *et al.*, 1994; Galzi *et al.*, 1991). The identity between human and rat deduced amino acid sequences in this domain is 93% for $\alpha3$ and 94% for $\alpha7$ subunits (Elliott *et al.*, 1996). Our observations with h$\alpha3\beta2$, h$\alpha3\beta4$ and h$\alpha7$ nAChRs suggest that the divergence in molecular structure between human and rat nAChR subunits $\alpha3$ and $\alpha7$ may account for the altered pharmacological properties of their assembled multimeric receptors.

The Hill slope values we obtained for some agonists in $\beta2$-containing hnAChRs are lower than those obtained for $\beta4$-containing hnAChRs. Lower Hill coefficients have been reported for nicotinic agonists in rat $\alpha3\beta2$ compared to rat $\alpha3\beta4$ nAChRs expressed in *Xenopus* oocytes (Cachelin and Jaggi, 1991; Covernton *et al.*, 1994; Cohen *et al.*, 1995). It is possible that nAChRs containing $\beta2$ subunits desensitize more rapidly than $\beta4$-containing receptors and that this desensitization accounts for the lower Hill coefficient estimates; however, typically faster decay rates were observed in h$\alpha3\beta2$ nAChRs than in h$\alpha2\beta2$ or h$\alpha4\beta2$ nAChRs, and yet comparable Hill values were obtained in these subtypes. Alternatively, the differences in Hill coefficients may reflect different interactions between $\beta2$ and $\beta4$ subunits with α subunits, determining the cooperativity of the assembled receptors, as proposed by Cohen *et al.* (1995).

Sensitivity to block by nicotinic receptor antagonists. Recombinant hnAChRs are inhibited by the nicotinic receptor antagonists MEC, DHβE and d-Tubo, and distinct relative sensitivities to these antagonists were observed among the seven hnAChRs subunit combinations. Receptors containing $\alpha4$ subunits, h$\alpha4\beta2$ and h$\alpha4\beta4$, were the only hnAChRs tested that display a higher sensitivity to DHβE than to d-Tubo. This result underscores the relevance of α subunits in determining the antagonist profile of these receptors. Human $\alpha4\beta2$ and h$\alpha4\beta4$ nAChRs can be differentiated by their sensitivity to MEC, with h$\alpha4\beta4$ being more

TABLE 2
Inhibition of agonist-induced currents in recombinant hnAChRss by DHβE and d-Tubo

	hα2β2	hα2β4	hα3β2	hα3β4	hα4β2	hα4β4	hα7
DHβE[a]							
K_b mean [b]	0.85 μM	3.61 μM	1.62 μM	13.77 μM	0.11 μM	0.01 μM	19.59 μM
(−S.D., +S.D.)[b]	(0.78, 0.93)	(1.65, 7.90)	(0.89, 2.94)	(11.34, 16.73)	(0.09, 0.14)	(0.01, 0.04)	(11.1, 34.57)
n_H (mean ± S.E.M.)	0.94 ± 0.09	0.99 ± 0.12	1.33 ± 0.26	0.94 ± 0.09	0.58 ± 0.05	0.88 ± 0.10	1.12 ± 0.11
(*N*)	(3)	(4)	(5)	(4)	(4)	(5)	(3)
d-Tubo[a]							
K_b mean[b]	1.36 μM[c]	4.24 μM	2.41 μM	2.24 μM[c]	3.16 μM[d]	0.21 μM[d]	3.10 μM[d]
(−S.D., +S.D.)[b]		(1.98, 9.09)	(1.89, 3.09)		(2.43, 4.13)	(0.07, 0.64)	(1.67, 5.75)
n_H (mean ± S.E.M.)	0.68[c]	0.81 ± 0.09	1.09 ± 0.14	1.03[c]	0.74 ± 0.04	0.95 ± 0.11	1.69 ± 0.30
(*N*)	(5)	(6)	(5)	(4)	(4)	(3)	(6)

[a] The agonist and dose used for each hnAChRs subunit combination are indicated in the text.
[b] Geometric mean values (see "Methods").
[c] A different group of oocytes was tested with each antagonist dose, and therefore, only one curve was fitted to the (averaged) data points (see "Methods").
[d] The difference in potency between d-Tubo and DHβE was statistically significant ($P < .01$).

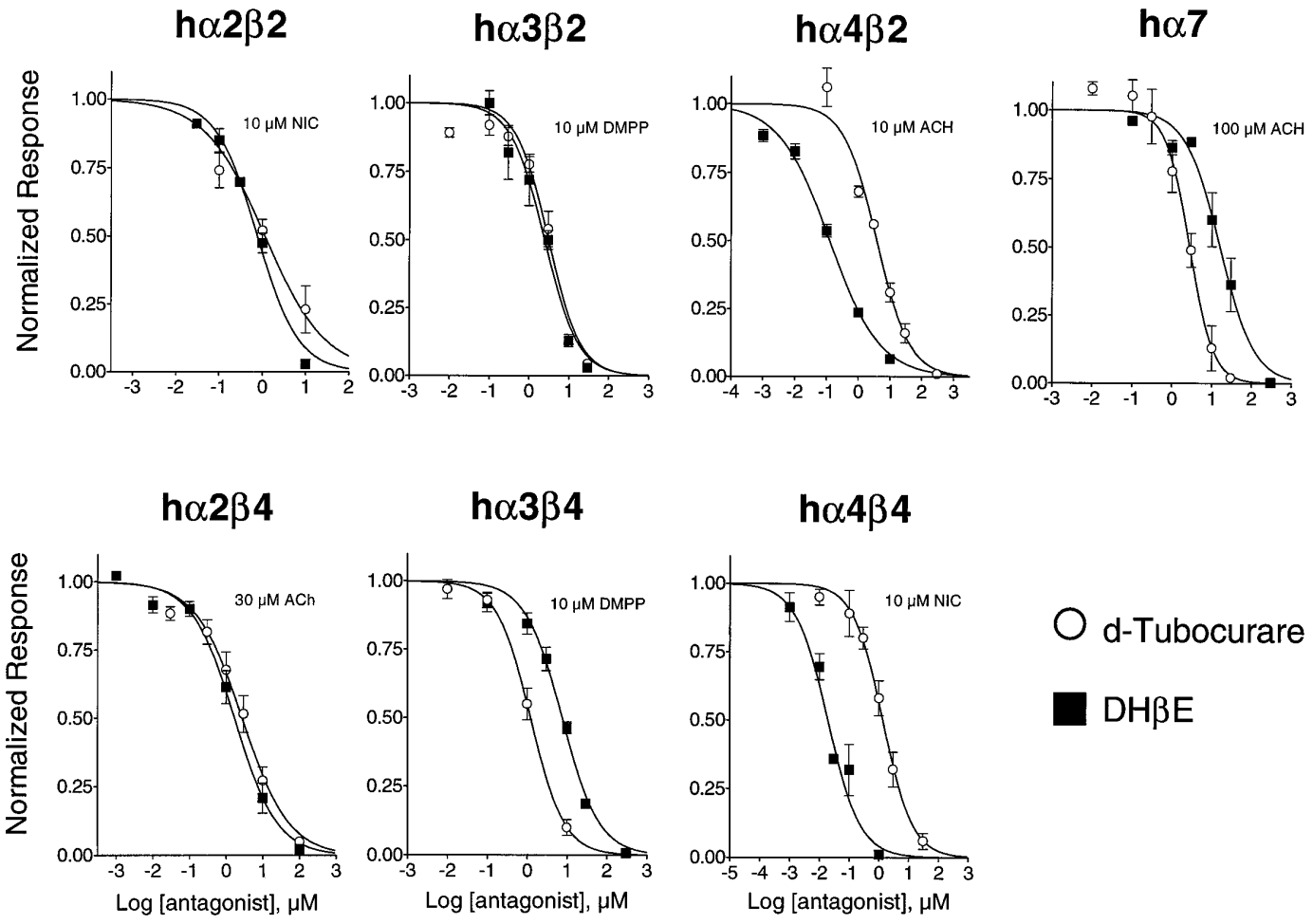

Fig. 7. The relative potency of the nicotinic receptor antagonists d-Tubo and DHβE differs among recombinant hnAChRs. Dose-response curves (fitted by nonlinear regression to the Hill equation, see "Methods") for d-Tubo and DHβE on all seven hnAChRs. Response amplitudes recorded upon the coapplication of either of these antagonists and a nicotinic agonist (indicated on the right of each plot), were normalized to the current amplitude elicited by the agonist alone. Data points represent the mean ± S.E.M. of the responses observed in three to six oocytes. The difference in potency between d-Tubo and DHβE was statistically significant for hα4β2, hα4β4 and hα7 ($P < .05$, Mann-Whitney); hα3β4 nAChRs cannot be tested for significance (see "Methods").

sensitive than hα4β2 nAChRs. Conversely, hα2β2, hα4β2 and hα7 nAChRs, which display a similar sensitivity to MEC, show different sensitivity profiles to DHβE and d-Tubo: hα2β2 displays a similar sensitivity to these two antagonists, hα4β2 is more sensitive to DHβE than d-Tubo, and hα7 is more sensitive to d-Tubo than DHβE.

Our K_b estimate for DHβE on hα3β4 nAChRs is ≈9-fold larger than that of hα3β2, and this difference is statistically

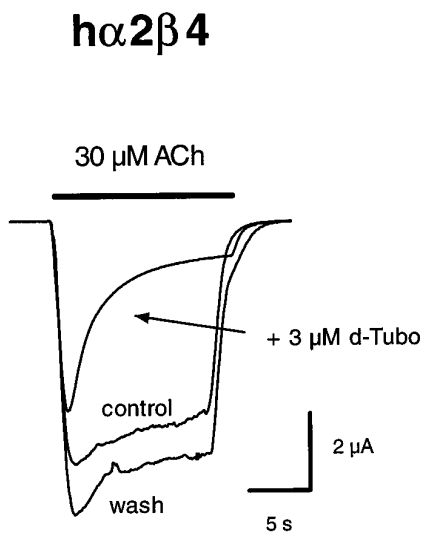

Fig. 8. The kinetics of agonist-induced currents in h$\alpha 2\beta 4$ nAChRs are altered by coapplication with submaximal doses of d-Tubo. Current responses elicited in an oocyte expressing h$\alpha 2\beta 4$ nAChRs by ACh in the absence (control), in the presence (arrow) and after washout of 3 μM d-Tubo (wash). Holding membrane potential -80 mV.

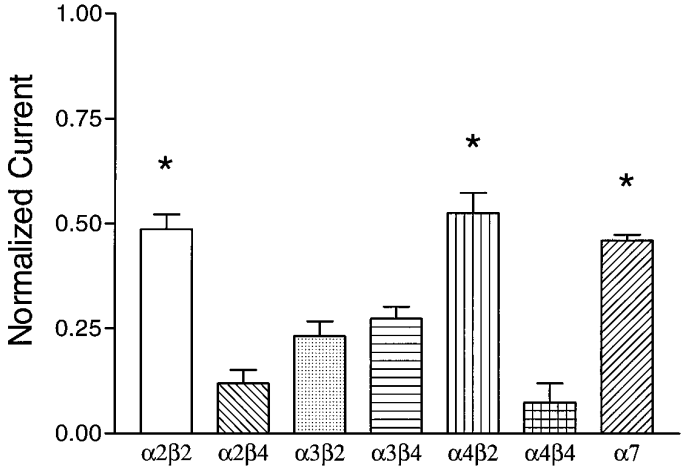

Fig. 9. Amplitude of the responses (mean ± S.E.M.) elicited by nicotinic agonists in the presence of 3 μM MEC as a fraction of the response recorded in its absence (n = 3–10 oocytes/group). The nicotinic agonist used for each nAChRs subunit combination is indicated in table 1. The sensitivity to MEC observed in h$\alpha 4\beta 4$ is significantly different from that seen in hnAChR indicated by an asterisk ($P < .0001$, Kruskal-Wallis analysis of variance, followed by Dunn's test, $P < .05$,).

significant. These results agree with the higher sensitivity to DHβE reported for rat $\alpha 3\beta 2$, compared with rat $\alpha 3\beta 4$ nAChRs (Harvey and Luetje, 1996) and emphasize the importance of α/β subunit interactions in the determination of the pharmacological properties of nAChRs.

The activity of d-Tubo on h$\alpha 2\beta 4$ and h$\alpha 4\beta 4$ suggests that a noncompetitive block mechanism is also involved in the inhibition of agonist-induced currents. It is also possible, however, that slower binding kinetics of d-Tubo to the agonist recognition site, compared to that of the agonist, contribute to our observations. Further studies are required to determine the mechanism of action of d-Tubo on these and other hnAChRs subunit combinations. This putative noncompetitive action of d-Tubo may compromise the utility of the K_b transformation of the IC_{50} values. A noncompetitive antagonism by d-Tubo has been reported for recombinant chick $\alpha 7$ nAChRs expressed in *Xenopus* oocytes (Bertrand *et al.*, 1992b), and a voltage-dependent block by d-Tubo (but not DHβE) has been observed in native rat nAChRs (Mulle *et al.*, 1991).

The sensitivity to DHβE appears different between human (K_b = 19.6 μM) and chick $\alpha 7$ nAChRs (1.6 μM; 100 μM ACh as agonist, Bertrand *et al.*, 1992b). Our K_b estimate for h$\alpha 7$ of 3.10 μM for d-Tubo was somewhat higher than the IC_{50} value of 0.7 μM estimate reported by Peng *et al.* (1994) for h$\alpha 7$ nAChRs; this may partly be due to the lower agonist concentration used in their study (30 μM NIC). These IC_{50} values are comparable to the estimated IC_{50} of 0.55 μM reported for rat $\alpha 7$ (Seguela *et al.*, 1993). No large differences were found in the sensitivities to MEC between h$\alpha 2\beta 2$ and h$\alpha 4\beta 2$ and their rat homologs. An IC_{50} of about 3 μM for MEC may be estimated for these hnAChRs, which is comparable to the IC_{50} of 1 μM estimated for their rat homologs (Connolly *et al.*, 1992).

The pharmacological profile of the different hnAChR subunit combinations observed in this study may help in the determination of the molecular composition of native nAChRs involved in agonist-induced responses in human cells. In particular, the agonist profile reported for the SH-SY5Y human neuroblastoma cell line using the Rb^+ flux assay (Lukas *et al.*, 1993) indicates that hnAChRs containing $\alpha 3\beta 4$ subunits significantly contribute to the functional nAChR pool in these cells.

Taken together, our data indicate that recombinant human nAChRs display unique pharmacological properties that are determined by their α and β subunits. Also, some pharmacological differences are apparent between human and rat (and chick) homologs. The distinct agonist/antagonist selectivity profiles observed for recombinant hnAChRs demonstrate the potential for discovery and development of subtype selective nicotinic ligands. Furthermore, the differences between the pharmacological properties of human and rat recombinant nAChRs underscores the importance of screening human nAChRs for the identification and development of nAChR ligands as potential therapeutic drugs.

Acknowledgments

The authors thank Kelly J. Berckhan for technical assistance, Dr. Janis Corey-Naeve and Dr. Kenneth Stauderman for helpful comments and critical review of a previous version of this manuscript.

References

Alkondon, M. and Albuquerque, E. X.: Diversity of nicotinic acetylcholine receptors in rat hippocampal neurons. I. Pharmacological and functional evidence for distinct structural subtypes. J. Pharmacol. Exp. Ther. **265:** 1455–1473, 1993.

Anand, R., Conroy, W. G., Schoepfer, R., Whiting, P., Lindstrom, J.: Neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes have a pentameric quaternary structure. J. Biol. Chem. **266:** 11192–11198, 1991.

Anand, R. and Lindstrom, J.: Nucleotide sequence of the human nicotinic acetylcholine receptor $\beta 2$ subunit gene. Nucleic Acids Res. **18:** 4272, 1990.

Baron, J. A.: Cigarette smoking and Parkinson's disease. Neurology **36:** 1490–1496, 1986.

Bertrand, D., Devillers-Thiery, F., Revah, F., Galzi, J.-L., Hussy, N., Mullle, C., Bertrand, S., Ballivet, M. and Changeux, J. P.: Unconventional pharmacology of a neuronal nicotinic receptor mutated in the channel domain. Proc. Natl. Acad. Sci. U.S.A. **89:** 1261–1265, 1992a.

Bertrand, D., Bertrand, S. and Ballivet, M.: Pharmacological properties of the homomeric $\alpha 7$ receptor. Neurosci. Lett. **146:** 87–90, 1992b.

Cachelin, A. B. and Jaggi, R.: β subunits determine the time course of desensitization in rat neuronal nicotinic acetylcholine receptors. Pflugers Arch. **419:** 579–582, 1991.

Chini, B., Clementi, F., Hukovic, N. and Sher, E.: Neuronal-type alpha-

bungarotoxin receptors and the alpha 5-nicotinic receptor subunit gene are expressed in neuronal and nonneuronal human cell lines. Proc. Natl. Acad. Sci. U.S.A. **89:** 1572–1576, 1992.

CLARKE, P. B. S.: Nicotinic receptors and cholinergic neurotransmission in the central nervous system. Ann. N.Y. Acad. Sci. **757:** 73–83, 1995.

CLARKE, P. B. S. AND KUMAR, R.: The effects of nicotine on locomotor activity in non-tolerant and tolerant rats. Br. J. Pharmac. **78:** 329–337, 1983.

CLARKE, P. B. S., ROCHELLE, D. S., PAUL, S. M., PERT, C. B. AND PERT, A.: Nicotinic binding in rat brain: autoradiographic comparison of [^{3}H]acetylcholine, [^{3}H]nicotine, and [^{125}I]-α-bungarotoxin. J. Neurosci. **5:** 1307–1315, 1985.

COHEN, B. N., FIGL, A., QUICK, M. W., LABARCA, C., DAVIDSON, N. AND LESTER, H. A.: Regions of β2 and β4 responsible for differences between the steady state dose-response relationships of the α3β2 and α3β4 neuronal nicotinic receptors. J. Gen. Physiol. **105:** 745–764, 1995.

CONNOLLY, J., BOULTER, J. AND HEINEMANN, S. F.: Alpha$_{4-2}$ beta$_2$ and other nicotinic acetylcholine receptor subtypes as targets of psychoactive and addictive drugs. Br. J. Pharmacol. **105:** 657–666, 1992.

CONNOLLY, J. G., GIBB, A. J. AND COLQUHOUN, D.: Heterogeneity of neuronal nicotinic acetylcholine receptors in thin slices of rat medial habenula. J. Physiol. (Lond.) **484:** 87–105, 1995.

COOPER, E., COUTURIER, S. AND BALLIVET, M.: Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. Nature **350:** 235–238, 1991.

COUTURIER, S., BERTRAND, D., MATTER, J. M., HERNANDEZ, M. C., BERTRAND, S., MILLAR, N., VALERA, S., BARKAS, T. AND BALLIVET, M.: A neuronal nicotinic acetylcholine receptor subunit (alpha 7) is developmentally regulated and forms a homo-oligomeric channel blocked by alpha-BTX. Neuron **5:** 847–856, 1990.

COVERNTON, P. J. O., KOJIMA, H., SIVILOTTI, L. G., GIBB, A. J. AND COLQUHOUN, D.: Comparison of neuronal nicotinic receptors in rat sympathetic neurones with subunit pairs expressed in *Xenopus* oocytes. J. Physiol. (Lond.) **481:** 27–34, 1994.

DINELEY-MILLER, K. AND PATRICK, J.: Gene transcripts for the nicotinic acetylcholine receptor subunit, beta4, are distributed in multiple areas of the rat central nervous system. Brain Res. Mol. Brain Res. **16:** 339–344, 1992.

DOUCETTE-STAMM, L., MONTEGGIA, L. M., DONNELLY-ROBERTS, D., WANG, M. T., LEE, J., TIAN, J. AND GIORDANO, T.: Cloning and sequence of the human α7 nicotinic acetylcholine receptor. Drug Dev. Res. **30:** 252–256, 1993.

ELGOYHEN, A. B., JOHNSON, D. S., BOULTER, J., VETTER, D. E. AND HEINEMANN, S.: α9: An acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell **79:** 705–715, 1994.

ELLIOTT, K. J., ELLIS, S. B., BERCKHAN, K. J., URRUTIA, A., CHAVEZ-NORIEGA, L. E., JOHNSON, E. C., VELICELEBI, G. AND HARPOLD, M.: Comparative structure of human neuronal α2-α7 and β2-β4 nicotinic acetylcholine receptor subunits and functional expression of the α2, α3, α4, α7, β2 and β4 subunits. J. Mol. Neurosci. **7:** 217–228, 1996.

FORNASARI, D., CHINI, B., TARRONI, P. AND CLEMENTI, F.: Molecular cloning of human neuronal nicotinic receptor alpha 3-subunit. Neurosci. Lett. **111:** 351–356, 1990.

GALZI, J.-L., BERTRAND, D., DEVILLERS-THIERY, A., REVAH, F., BERTRAND, S. AND CHANGEUX, J.-P.: Functional significance of aromatic amino acids from three peptide loops of the α7 neuronal nicotinic receptor site investigated by site-directed mutagenesis. FEBS Lett. **294:** 198–202, 1991.

GERZANICH, V., ANAND, R. AND LINDSTROM, J.: Homomers of alpha 8 and alpha 7 subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. Mol. Pharmacol. **45:** 212–220, 1994.

GERZANICH, V., PENG, X., WANG, F., WELLS, G., ANAND, R., FLETCHER, S. AND LINDSTROM, J.: Comparative pharmacology of epibatidine: A potent agonist for neuronal nicotinic acetylcholine receptors. Mol. Pharmacol. **48:** 774–782, 1995.

GOPALAKRISHNAN, M., BUISSON, B., TOUMA, E., GIORDANO, T., CAMPBELL, J. E., HU, I. C., DONNELY-ROBERTS, D., ARNERIC, S. P., BERTRAND, D. AND SULLIVAN, J. P.: Stable expression and pharmacological properties of the human α_7 nicotinic acetylcholine receptor. Eur. J. Pharmacol. Mol. Pharmacol. **290:** 237–246, 1995.

GOPALAKRISHNAN, M., MONTEGGIA, L. M., ANDERSON, D. J., MOLINARI, E. J., PIATTONI-KAPLAN, M., DONNELLY-ROBERTS, D., ARNERIC, S. P. AND SULLIVAN, J. P.: Stable expression, pharmacologic properties and regulation of the human neuronal nicotinic acetylcholine $\alpha_4\beta_2$ receptor. J. Pharmacol. Exp. Ther. **276:** 289–297, 1996.

HARVEY, S. C. AND LUETJE, C. W.: Determinants of competitive antagonist sensitivity on neuronal nicotinic receptor β subunits. J. Neurosci. **16:** 3798–3806, 1996.

HUSSY, N., BALLIVET, M. AND BERTRAND, D.: Agonist and antagonist effects of nicotine on chick neuronal nicotinic receptors are defined by alpha and beta subunits. J. Neurophysiol. **72:** 1317–1326, 1994.

JONES, M. V. AND WESTBROOK, G.: The impact of receptor desensitization on fast synaptic transmission. Trends Neurosci. **19:** 96–101, 1996.

KOZAK, M.: An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15:** 8125–8148, 1987.

LEFF., P. AND DOUGALL, G.: Further concerns over Cheng-Prusoff analysis. Trends Pharmacol. Sci. **14:** 110–112, 1993.

LEVIN, E. D., CHRISTOPHER, N. C., BRIGGS, S. J. AND ROSE, J. E.: Chronic nicotine reverses working memory deficits caused by lesions of the fimbria or medial basalocortical projection. Cognitive Brain Res. **1:** 137–143, 1993.

LUETJE, C. W. AND PATRICK, J.: Both alpha- and beta-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. J. Neurosci. **11:** 837–845, 1991.

LUKAS, R. J., NORMAN, S. A. AND LUCERO, L.: Characterization of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y human neuroblastome clonal line. Mol. Cell. Neurosci. **4:** 1–12, 1993.

MACONOCHIE, D. J AND KNIGHT, D. E.: A study of the bovine adrenal chromaffin nicotinic receptor using patch clamp and concentration jump techniques. J. Physiol. (Lond.) **454:** 129–153, 1992.

MAHAFFY, L. S., STAUDERMAN, K. A., CHAVEZ-NORIEGA, L. E., CRONA, J. AND COREY-NAEVE, J.: HEK293 cells stably expressing recombinant human neuronal nicotinic acetylcholine receptors α3β2 and α4β2 display differential sensitivity to external Ca^{2+}. Soc. Neurosci. Abstr. **22:** p. 1526, 1996.

MCGEHEE, D. S. AND ROLE, L. W.: Physiological diversity of nicotinic acetylcholine receptors expressed by vertebrate neurons. Annu. Rev. Physiol. **57:** 521–546., 1995.

MILEDI, R. AND PARKER, I.: Chloride current induced by injection of calcium into Xenopus oocytes. J. Physiol. (Lond.) **357:** 173–183, 1984.

MOSS, D. E., MANDERSCHEID, P. Z., MONTGOMERY, S. P., NORMAN, A. B. AND SANBERG, P. R.: Nicotine and cannabinoids as adjuncts to neuroleptics in the treatment of Tourette syndrome and other motor disorders. Life Sci. **44:** 1521–1525, 1989.

MULLE, C., VIDAL, C., BENOIT, P. AND CHANGEUX, J. P.: Existence of different subtypes of nicotinic acetylcholine receptors in the rat habenulo-interpeduncular system. J. Neurosci. **11:** 2588–2597, 1991.

MULLE, C., LENA, C. AND CHANGEUX, J.-P.: Potentiation of nicotinic receptor response by external calcium in rat central neurons. Neuron **8:** 937–945, 1992.

NEWHOUSE, P. A., SUNDERLAND, T., TARIOT, P. N., BLUMHARDT, C. L., WEINGARTNER, H., MELLOW, A. AND MURPHY, D. L.: Intravenous nicotine in Alzheimer's disease: A pilot study. Psychopharmacology **95:** 171–175, 1988.

NORDBERG, A.: Human nicotinic receptors—Their role in aging and dementia. Neurochem. Int. **25:** 93–97, 1994.

PAPKE, R. L.: The kinetic properties of neuronal nicotinic receptors: Genetic basis of functional diversity. Prog. Neurobiol. **41:** 509–531, 1993.

PENG, X., KATZ, M., GERZANICH, V., ANAND, R. AND LINDSTROM, J.: Human alpha 7 acetylcholine receptor: cloning of the alpha 7 subunit from the SH-SY5Y cell line and determination of pharmacological properties of native receptors and functional alpha 7 homomers expressed in *Xenopus* oocytes. Mol. Pharmacol. **45:** 546–554, 1994.

RINNE, J. O., MYLLYKYLÄ, T., LÖNNBERG, P. AND MARJAMÄKI, P.: A postmortem study of brain nicotinic receptors in Parkinson's and Alzheimer's disease. Brain Res. **547:** 167–170, 1991.

RUBBOLI, F., COURT, J. A., SALA, C., MORRIS, C., PERRY, E. AND CLEMENTI, F.: Distribution of neuronal nicotinic receptor subunits in human brain. Neurochem. Int. **25:** 69–71, 1994.

SARGENT, P. B.: The diversity of neuronal nicotinic acetylcholine receptors. Annu. Rev. Neurosci. **16:** 403–443, 1993.

SÉGUÉLA, P., WADICHE, J., DINELEY-MILLER, K., DANI, J. A. AND PATRICK, J. W.: Molecular cloning, functional properties, and distribution of rat brain α_7: A nicotinic cation channel highly permeable to calcium. J. Neurosci. **13:** 596–604, 1993.

STEINLEIN, O. K., MULLEY, J. C., PROPPING, P., WALLACE, R. H., PHILLIPS, H. A., SUTHERLAND, G. R., SCHEFFER, I. E. AND BERKOVIC, S. F.: A missense mutation in the neuronal nicotinic acetylcholine receptor α4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. Nature Genet. **11:** 201–203, 1995.

TARRONI, P., RUBBOLI, F., CHINI, B., ZWART, R., OORTGIESEN, M., SHER, E. AND CLEMENTI, F.: Neuronal-type nicotinic receptors in human neuroblastoma and small-cell lung carcinoma cell lines. FEBS Lett. **312:** 66–70, 1992.

VERNINO, S., AMADOR, M., LUETJE, C. W., PATRICK, J. AND DANI, J. A.: Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. Neuron **8:** 127–134, 1992.

WADA, E., WADA, K., BOULTER, J., DENERIS, E., HEINEMANN, S., PATRICK, J. AND SWANSON, L. W.: Distribution of Alpha2, Alpha3, Alpha4 and Beta2 neuronal nicotinic receptor subunit mRNAs in the central nervous system: A hybridization histochemical study in the rat. J. Compar. Neurol. **284:** 314–335, 1989.

WILLOUGHBY, J. J., NINKINA, N. N., BEECH, M. M., LATCHMAN, D. S. AND WOOD, J. N.: Molecular cloning of a human neuronal nicotinic acetylcholine receptor beta 3-like subunit. Neurosci. Lett. **155:** 136–139, 1993.

WONG, E. T., HOLSTAD, S. G., MENNERICK, S. J., HONG, S. E., ZORUMSKI, C. F. AND ISENBERG, K. E.: Pharmacological and physiological properties of a putative ganglionic nicotinic receptor, α3β4, expressed in transfected eukaryotic cells. Mol. Brain Res. **28:** 101–109, 1995.

ZHANG, Z. W., VIJAYARAGHAVAN, S. AND BERG, D. K.: Neuronal acetylcholine receptors that bind alpha-bungarotoxin with high affinity function as ligand-gated ion channels. Neuron **12:** 167–177, 1994.

Send reprint requests to: Dr. Laura E. Chavez-Noriega, SIBIA Neurosciences, Inc., 505 Coast Boulevard South, Suite 300, La Jolla, CA 92037.