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Ligand Binding and Activation of Rat Nicotinic $\alpha4\beta2$ Receptors Stably Expressed in HEK293 Cells

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ABSTRACT

HEK293 cells were stably transfected with rat neuronal nicotinic $\alpha4$ and $\beta2$ subunits. Binding of tritiated cytisine and nicotine to cell homogenates revealed the presence of a single class of high-affinity sites (dissociation constants 0.1 nM and 0.4 nM, respectively). Activation of nicotinic receptors was studied using whole-cell patch clamp methods, and acetylcholine, nicotine, dimethylphenylpiperazinium, and cytisine all produced a conductance increase. Responses desensitized to prolonged applications, at both positive and negative membrane potentials. The conductance was strongly rectifying, and outward currents were essentially absent. Responses were maximal at about 2 mM external calcium ion concentration and were reduced by about one-half at either nominally 0 or 10 mM external calcium. Di-hydro-β-erythroidine blocked physiological responses to acetylcholine and nicotine (IC_{50}, 2.5 nM), and reduced cytisine binding in a competitive manner (K_i 20 nM). Physostigmine enhanced the response to low concentrations of acetylcholine or nicotine. The anesthetic steroid (+)-3α-hydroxy-5α-androstane-17β-carbonitrile blocked responses to acetylcholine (IC_{50}, 1.3 μM), but had no effect on cytisine binding at a concentration of 30 μM. The binding properties of the receptors are those expected for rat neuronal nicotinic receptors composed of $\alpha4$ and $\beta2$ subunits. The pharmacological properties indicate that the responsiveness of the receptors may be allosterically enhanced or inhibited.

A number of subunits for nicotinic receptors are expressed by cells of the nervous system (reviewed in McGehee and Role, 1995; Brioni et al., 1997), including several subunits most similar to the muscle α subunit ($\alpha2 - \alpha9$) and others termed β subunits ($\beta2 - \beta4$). A major type of neuronal nicotinic receptor in the mammalian and avian brain is composed of the $\alpha4$ and $\beta2$ subunits (Whiting et al., 1987a; Schoepfer et al., 1988; Flores et al., 1992; Picciotto et al., 1995) and forms a protein with a very high affinity for agonists such as nicotine and cytisine (reviewed in Brioni et al., 1997). The functional role of the neuronal nicotinic $\alpha4\beta2$ receptor is not well understood; at present, it seems likely that it may modulate the release of neurotransmitter as a consequence of presynaptic localization (reviewed in McGehee and Role, 1996). Consistent with this idea is the finding that genetically altered mice in which the $\beta2$ subunit has been knocked out are grossly normal behaviorally, although they show changes in some forms of learning (Picciotto et al., 1995). It has been found, however, that the numbers of $\alpha4\beta2$ receptors are increased by chronic nicotine exposure in animals (Flores et al., 1992), and the numbers of high-affinity nicotine-binding sites are increased in the brains of humans who smoked and decreased in patients with Alzheimer's disease (reviewed in Brioni et al., 1997). It is also of note that mutations of the human $\alpha4$ subunit have been identified in families showing autosomal dominant nocturnal frontal lobe epilepsy (Steinlein et al., 1997).

As a step toward better understanding of the functional properties of this nicotinic receptor, we have stably expressed rat nicotinic $\alpha4$ and $\beta2$ subunits in human epithelial cells (HEK293) and characterized the binding and functional properties of the receptors.

Materials and Methods

Unless otherwise noted, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). (+)-3α-Hydroxy-5α-androstane-17β-carbonitrile (+ACN) was a gift from Dr. D. Covey (Washington University School of Medicine, St. Louis, MO). All values are presented and shown in graphs as the arithmetic mean ± 1 S.D., based on n observations.

Production of HEK293 Cells Stably Transfected with Rat Neuronal Nicotinic $\alpha4$ and $\beta2$ Subunits. HEK293 cells (CRL-1573: American Tissue Culture Collection, Gaithersburg MD) were maintained in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 (1:1, also containing 5 mM HEPES), with 10% fetal bovine serum (Hyclone, Logan UT), penicillin (100 u/ml) and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2

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ABBREVIATIONS: +ACN, (+)-3α-hydroxy-5α-androstane-17β-carbonitrile; PBS, phosphate-buffered saline; HB, homogenate buffer; DMSO, dimethyl sulfoxide; dHβE, di-hydro-β-erythroidine; mAb, monoclonal antibody; GABA, γ-aminobutric acid.

at 37°C. They were transfected by electroporation with expression constructs for the rat α4 and β2 subunits, and transfected cells were initially selected by growth in medium containing G418 (450 μg/ml; GIBCO, Grand Island, NY). Drug-resistant cells were maintained in G418, and then repeatedly immunoselected with monoclonal antibody (mAb) 270, which binds to an epitope on the extracellular surface of the β2 subunit (Whiting et al., 1987b). Selection was performed with magnetic beads with covalently attached sheep anti-rat antibody (Dynabeads M-450; Dynal, Lake Success, NY). Beads were prepared by mixing 1 ml of purified mAb 270 (1 mg/ml) with 1 ml of resuspended bead solution and 1 ml of phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 9.6 mM PO_4, pH 7.4) in a 15-ml sterile conical tube and rocking at 4°C for 30 min. The beads were washed twice by centrifugation and resuspension in PBS, and finally resuspended in 1 ml of PBS. To select the cells, 0.5 ml of a single-cell suspension (4×10^7 cells/ml) and 30 μl of freshly prepared sterile beads were mixed by gentle trituration and then incubated on ice for 15 min. The mixture was diluted by the addition of 10 ml of PBS, and cells were collected using a magnetic stand (Dynal). The beads (with attached cells) were washed a total of six times with PBS and then plated in a 60-mm tissue culture dish in growth medium. After overnight incubation, the dishes were washed twice with growth medium to remove beads. The cells used in these studies had been sequentially selected 6 to 10 times, but had not been cloned. They will be termed HN42 cells. mAb 270 was purified from serum-free growth medium from hybridoma cells (HB-189; ATCC).

The cDNAs for the rat neuronal nicotinic subunits were kindly provided by Dr. J. Patrick (Baylor College of Medicine) (rat α4 subunit HYA23–1E(1), Goldman et al., 1987; β2 subunit PCX49(1), Deneris et al., 1988). They were transferred to pcDNA3 (Invitrogen, San Diego, CA) for expression.

Binding Assays. Cells were grown in 10-cm culture dishes for 4 to 6 days and harvested by rinsing with PBS, then releasing the cells with PBS containing 5 mM EDTA. Cells from 10 dishes were pooled and pelleted by centrifugation. The pellet was washed once with PBS by resuspension and pelleting. The washed pellet was resuspended and cell number was estimated in a hemocytometer. The suspension was centrifuged, the supernatant removed, and the cell pellet frozen at −80°C.

The cell homogenate was prepared by thawing the pellet in 5 ml of PBS containing 5 mM EDTA and 300 μM phenylmethylsulfonyl fluoride. The pellet was suspended by trituration and then cells were broken by sonication (Branson 250 Sonifier, about 10 s on a setting of 2). The suspension was centrifuged at 13,000*g* (Beckman JA20 rotor, 13,000 rpm) for 20 min at 4°C, and the supernatant was removed. The pellet was resuspended in homogenate buffer (HB) (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$) by sonication. The mixture was centrifuged again and then the final pellet was suspended in 1 ml of HB. Total protein in the final homogenate was assayed by the bicinchonic acid method (Pierce Chemical Co., Rockford IL), with bovine serum albumin as the standard.

Binding assays were performed in HB. The cell homogenate was thawed at 4°C, and diluted in cold HB to an appropriate final concentration. Nonradioactive displacing drugs were added, mixed and incubated at 4°C for about 15 min. Then [^{3}H]nicotine (64 Ci/mmol; DuPont NEN) or [^{3}H]cytisine (40 Ci/mmol; DuPont NEN) was added to the desired final concentration, and tubes were vortexed, and placed on ice for 90 min. Binding in each experiment was determined as the mean of duplicate tubes. Most assays were performed in polypropylene tubes (12 × 75 mm), but studies involving +ACN and/or dimethyl sulfoxide (DMSO) were performed in glass tubes. Controls showed indistinguishable binding in the two types of tubes. The assays were performed with a constant amount of protein per tube (50 μg), containing about 21 fmol of sites (±7 fmol, 13 determinations of specific binding at 10 nM cytisine). The following final volumes used were: 0.01 to 0.3 nM ligand, 3 ml; 1 nM, 1.5 ml; 3 nM, 0.5 ml; 10 to 100 nM, 0.2 ml.

After incubation, the cell fragments were harvested by filtration (Brandel, Gaithersberg MD) on GF/C filter paper which had been presoaked with polyethyline imine (0.5% w/v in water; Schwartz et al., 1982). The filters were rinsed three times with HB. Filter discs were transferred to scintillation vials and allowed to stand overnight at room temperature in EconoSolve (Research Products International, Mount Prospect IL) and then counted in a scintillation counter.

Specific binding was determined by subtracting the binding to homogenates prepared from untransfected HEK293 cells (see *Results*) after normalizing to the amount of protein in the homogenate. Binding curves were fit with SigmaPlot (SSPS, Chicago IL).

Electrophysiology. Cells were plated in 35- or 60-mm tissue culture dishes, and used within 4 days after plating. Recordings were made in an extracellular saline, most often composed of 140 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 5 mM KCl, 10 mM glucose, and 1 μM atropine sulfate, pH 7.3. When a different saline was used, the modifications are noted in *Results*. The internal solution was composed of 4 mM NaCl, 4 mM $MgCl_2$, 0.5 mM $CaCl_2$,, 10 mM HEPES, 5 mM EGTA, and 140 mM CsCl, pH 7.3. Standard methods were used to record currents in the whole cell configuration. Records were filtered with a four-pole Bessel filter (Frequency Devices, Haverhill, MA) and directly digitized by a PC clone computer with a TLC1 or Digidata interface (Axon Instruments, Foster City, CA). Drugs were dissolved in external solution and applied through a multiline perfuser (Fletcher and Steinbach, 1996) or a three-tube perfusion system (Maconochie and Knight, 1989). The multiline perfuser provided a solution change around cells attached to the culture substrate with a 10 to 90% time of about 200 ms, and the three-tube perfuser an exchange time of about 50 ms (data not shown, from the time course of changes in holding current when bath solutions with different ion concentrations were applied to cells). In either case, the cell was perfused with extracellular solution continuously between applications of agonists or other drugs.

+ACN was prepared as a 10 mM stock in DMSO, and diluted into extracellular solution. The highest concentration of DMSO in the final solution was 0.3% (42 mM). To avoid loss of +ACN or DMSO as a result of solubility in plastics, the perfusion system used glass syringe reservoirs, Teflon tubing, Teflon valves, and quartz perfuser tips.

The amplitude of a response was taken as the mean of a short interval centered at the peak response; drug effects were normalized to the interpolated value of controls taken before and after the test response. Current-voltage relationships were obtained with a ramp command potential generated in Clampex (Axon Instruments), which increased linearly from −100 mV to +100 mV over 2 s (0.1 mV/ms). Indistinguishable results were obtained with ramps from +100 mV to −100 mV (data not shown). The current-voltage relationships were obtained from the average of four traces taken during a single application of acetylcholine, with the average of eight control records (four preceding and four following) subtracted. To measure the onset rate for block by di-hydro-β-erythroidine (dHβE), dHβE was applied alone for a defined period, then the response was measured by immediately switching to an application of acetylcholine plus dHβE. The offset of block was measured by applying 100 nM dHβE for 10 s (producing full block), washing with extracellular solution for a defined period of time, and then testing the response. In either case, the response was normalized to the interpolated response to control applications before the application of dHβE and 80 s after the application of dHβE. +ACN was applied for 30 s and then the cell response was tested by immediately switching to a solution containing acetylcholine without +ACN or DMSO.

The response of a cell usually varied over time. Most often, responses initially increased, then decreased at longer times of recording ("ran down"). The decline was an experimental problem because it limited our ability to obtain data from experiments which required long series of drug applications or prolonged wash periods. In preliminary experiments, the decline was similar using acetylcholine or

nicotine as agonist and using whole cell or perforated patch recordings (data not shown). Run down, or perhaps accumulation in long-lived desensitized states, was increased by repeated applications of high concentrations of agonist. To reduce this, applications of high concentrations of agonists were separated by 60 s or more.

As already reported for responses from PC12 cells (Ifune and Steinbach, 1993), we also found that desensitization developed more rapidly with time of whole-cell recording. The more rapid development resulted from an increase in both the rate and the relative amplitude of the rapidly declining component, with a smaller change in the rate of the slower component. Because of the change in desensitization with time, we compared the desensitization seen when acetylcholine was applied at +50 mV to adjacent responses when acetylcholine was applied at −100 mV.

Results

Binding of Nicotine and Cytisine

Specificity of Cytisine Binding. The equilibrium binding was measured of cytisine to homogenates prepared from HEK293 cells stably transfected with rat $\alpha 4$ and $\beta 2$ nicotinic receptor subunits (HN42 cells). Nonspecific binding was determined from binding to homogenates prepared from the parental HEK293 cells and from binding to homogenates from HN42 cells in the presence of 100 μM nicotine. The two assays for nonspecific binding were similar (Fig. 1) and amounted to a small fraction of the total binding to HN42 cell homogenates. On average, the specific binding by 10 nM cytisine was better than 90% of the total (92% ± 2%; $n = 6$).

Nicotine (100 μM) completely blocked specific cytisine binding (Fig. 1), and the total number of sites for nicotine and cytisine were statistically indistinguishable. At 10 nM, the specific cytisine binding was 383 ± 73 fmol per mg of protein ($n = 6$) whereas that for nicotine was 544 ± 117 fmol per mg ($n = 2$) (from a two-tailed t test, $p = .09$). Using the average protein per cell, this would correspond to about 10,000 sites per cell. These data suggest that nicotine and cytisine bind to the same site, although separate sites showing strong negative allosteric interactions could produce similar results.

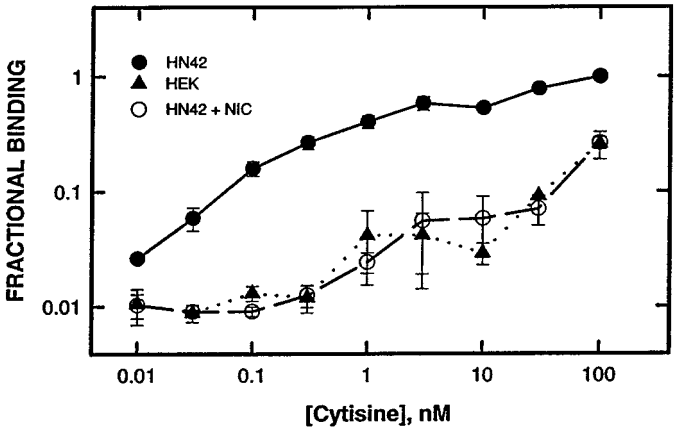

Fig. 1. Specificity of cytisine binding. The binding of cytisine was measured to homogenates prepared from HN42 cells (●). Nonspecific binding was determined with homogenates prepared from the parental HEK293 cells (▲) and by binding to homogenates from HN42 cells in the presence of 100 μM nicotine (○). The two assays for nonspecific binding are very similar at all concentrations of cytisine. The amount bound per milligram of protein was normalized to the maximal amount bound to HN42 cell homogenates in the absence of nicotine. The points show the mean of two independent experiments, each performed as duplicates, and error bars show 1 S. D. Note that both axes are logarithmically scaled.

Affinity of Cytisine and Nicotine Binding. Specific binding of cytisine and nicotine was saturable (Fig. 2). The curves through the data show fits of eq. 1:

$$Y = Y_{max}(\{[ligand]/K_h\}^{Nh})/(1 + \{[ligand]/K_h\}^{Nh}) \quad (1)$$

The apparent dissociation constants (K_h) are about 0.1 nM for cytisine (0.12 ± 0.05; $n = 3$) and 0.4 nM for nicotine (0.40 ± 0.01; $n = 2$), whereas the Hill coefficients (N_h) are close to 1 (0.97 ± 0.14 for cytisine and 0.99 ± 0.00 for nicotine).

Inhibition of Cytisine Binding. dHβE inhibited the binding of cytisine (Fig. 3). The curves through the data show fits of eq. 2:

$$Y = 1/(1 + \{[blocker]/IC_{50}\}^{Nh}) \quad (2)$$

As shown in Fig. 3, the concentration of dHβE required to reduce the amount bound by one half (IC_{50}) was larger at 3 nM cytisine than at 0.03 nM cytisine. The Hill slope was close to 1 in each case.

The apparent affinity (K_i) for dHβE was calculated from the measured IC_{50}, assuming competitive inhibition of binding at a single site, from eq. 3:

$$K_i = (IC_{50})/(1 + \{[Cyt]/K_{Cyt}\}) \quad (3)$$

The calculated K_i s are 25 nM (3 nM cytisine) and 18 nM (0.03 nM cytisine). These result indicate that dHβE is a formally competitive inhibitor of cytisine binding and suggest that dHβE is a competitive inhibitor of agonist binding to these receptors.

In one experiment, the ability of nicotine to prevent binding of 0.03 nM cytisine was examined. The estimated K_i for nicotine from this experiment was 0.43 nM, in good agreement with the K_h value from direct binding.

Electrophysiological Experiments (see below) indicated that the steroid +ACN blocks currents activated by acetylcholine or nicotine with an IC_{50} of about 2 μM. However, in

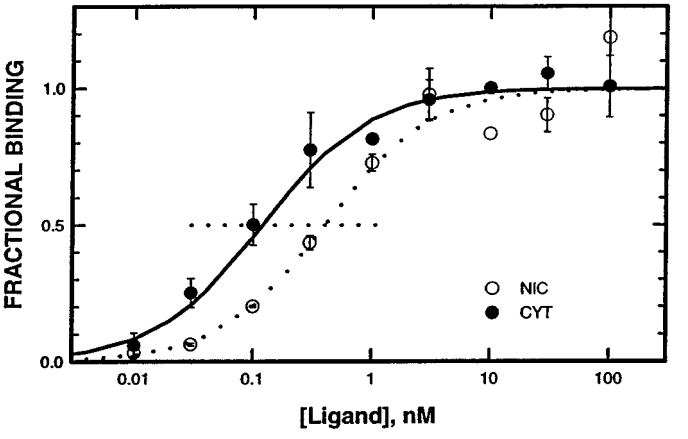

Fig. 2. Affinity of cytisine and nicotine binding. The specific binding of cytisine (● and solid line) and nicotine (○ and dotted line) to homogenates of HN42 cells saturates at high ligand concentrations. Nonspecific binding was determined with homogenates prepared from the parental HEK293 cells, and has been subtracted from the data shown. The curves show fits of eq. 1 to the data, and the horizontal dotted line shows half-maximal binding to indicate the K_h. The data are means of two or three separate binding experiments, each performed in duplicate. The data have been normalized to the binding measured at 10 nM of either ligand and then renormalized to the maximal fit binding. Parameters for the fit curves are given in the text.

contrast to the action of dHβE, +ACN did not reduce cytisine binding (Fig. 4, filled triangles). DMSO, the solvent used to prepare stock solutions of the steroid, did inhibit cytisine binding, albeit at high concentrations (data not shown; IC_{50} 156 mM, N_h 0.83; $n = 2$).

Physiological Responses

Activation and Desensitization by Nicotinic Agonists. HN42 cells responded to applications of nicotinic agonists (nicotine, acetylcholine, dimethylphenylpiperazinium, cytisine) with a conductance increase (Fig. 5). The concentration-response relationship was examined for acetylcholine. As shown in Fig. 5 (top panel), the relationship showed relatively low affinity and Hill slope. When fit with eq. 1, the estimate for K_h was 80 μM and for N_h was 0.7.

Nicotine appeared to be similar to acetylcholine in terms of ability to evoke currents. Cytisine appeared to activate more current than acetylcholine at very low concentrations, but the maximal evoked current was lower. The concentrations of cytisine or nicotine providing half-maximal activation were not determined, but appear to be greater than 30 nM for cytisine and 3 μM for nicotine. These concentrations are much greater than the values for K_h estimated from binding to homogenates, suggesting that the binding data reflect affinity for desensitized receptors.

Prolonged applications of nicotine (not shown) or acetylcholine produced desensitization of the response (Fig. 6A). Prolonged applications of 100 μM acetylcholine (20 s) resulted in full desensitization (residual fractional response at 20 s 0.015 ± 0.017 of peak; $n = 6$ cells). The decline in current to a 2-s application of 100 μM acetylcholine could be described by the sum of two exponentials declining to zero. Previous studies of neuronal nicotinic receptors have shown that different types of receptor show differing amounts of desensitization at positive potentials. Responses from rat sympathetic ganglion cells show no desensitization when cells are held at +50 mV (Mathie et al., 1990), whereas responses from rat PC12 pheochromocytoma cells show similar amounts of desensitization at −80 mV and +40 mV (Ifune and Steinbach, 1993). Studies of chicken subunits expressed in *Xenopus* oocytes indicated that the α4-nα1 combination showed no desensitization during a 4-s application of 50 μM acetylcholine at +40 mV, whereas the α3-nα1 combination showed complete desensitization at +40 mV during a 4-s application of 100 μM acetylcholine (Gross et al., 1991).

Desensitization at positive potentials cannot be directly examined, due the absence of measurable current (Fig. 7). Accordingly, we held cells at +50 for a period of time while 100 μM acetylcholine was applied, then jumped the potential to −100 mV to assay responses. The amplitude of the response immediately upon jumping to −100 mV was clearly reduced from the peak response at −100 mV, demonstrating that desensitization had occurred at +50 mV (Fig. 6B). However, there was consistently more current when the potential was returned to −100 mV than if the potential had been maintained at −100 mV throughout the application (Fig. 6B). Analysis of the data is made more complicated by the existence of two components in the desensitization time course (Fig. 6A). An initial insight can be gained by considering the relative currents when the potential is jumped to −100 mV, relative to the current if it is held at −100 mV for the entire application of acetylcholine. When the duration at +50 mV is only 250 ms the relative current is about 1.5 times the control, while when the duration at +50 mV is 1000 ms, the relative current is 1.8-fold the control. This suggests that the rapidly decaying component proceeds at about the same rate at both +50 mV and −100 mV (so the relative current after a brief exposure to acetylcholine at +50 mV is more modestly enhanced), whereas the slowly decaying component is more significantly slowed at +50 mV (so the relative current is more markedly enhanced). Further analysis of the data, assuming that only the rates (not the relative amplitudes) of the two components are altered at +50 mV, indicates that the rate for the fast component was decreased by only about 1.5-fold, whereas the rate for the slow component was decreased to a greater extent (5- to 10-fold). This analysis

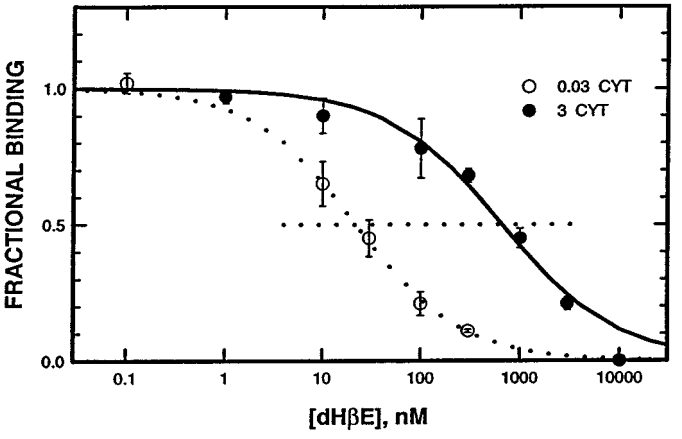

Fig. 3. Inhibition of cytisine binding by dHβE. The ability of increasing concentrations of dHβE to inhibit binding of 0.03 nM cytisine (○ and dotted line) or 3 nM cytisine (● and solid line) is shown. The amount bound is normalized to the binding in the absence of dHβE. The curves show fits of eq. 2 to the data. The parameters fit were IC_{50}: 656 ± 201 nM (3 nM cytisine) and 23 ± 8 nM (0.03 nM cytisine; $n = 2$ in each case). The Hill coefficients were: 0.75 ± 0.17 (3 nM cytisine) and 0.84 ± 0.09 (0.03 nM cytisine).

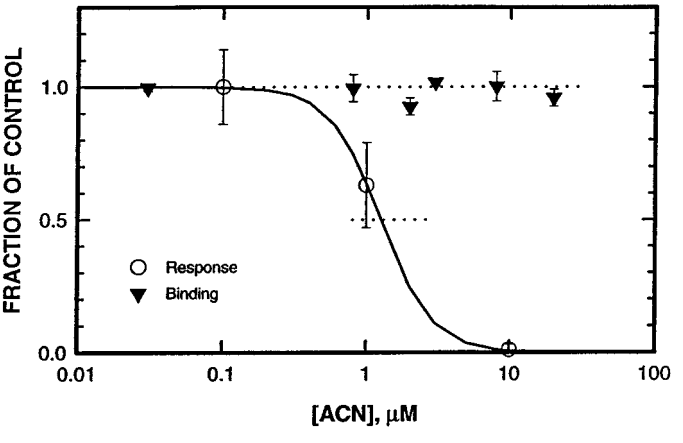

Fig. 4. The steroid +ACN has no effect on cytisine binding but blocks acetylcholine-elicited currents. The binding of cytisine (0.03 nM cytisine) is shown at various concentrations of the steroid analog +ACN (▼, mean of two experiments). +ACN was dissolved in DMSO before addition to the binding buffer; the final concentration of DMSO was 14 mM (0.1% V/V) for all concentrations of +ACN. The binding data were normalized to the binding seen in the presence of this concentration of DMSO, but no +ACN. The ○ show the relative response to 1 μM acetylcholine in the presence of +ACN (data from 3–5 cells), and the solid line shows the fit of eq. 2 to the data (IC_{50} 1.3 μM, N_h 2.4).

confirms the initial impression that with these cells, desensitization does occur at +50 mV, but at a reduced rate compared with −100 mV.

Current-Voltage Relationship and Sensitivity to Extracellular Calcium. The response to agonists was strongly rectifying, such that the outward conductance was essentially zero (Fig. 7). There was no indication of an increase in conductance at large positive membrane potentials (e.g., Ifune and Steinbach, 1990). Others have reported that receptors formed from human (Buisson et al., 1996) and chicken (Whiting et al., 1991) $\alpha4\beta2$ subunits show similar rectification properties.

The effect of altered extracellular calcium concentration on the response to 1 μM nicotine was assessed. Compared with the response with 2 mM external calcium, an increase to 10 mM reduced the response to 0.39 ± 0.10 times control (n = 4), and removal of calcium from the solution also reduced the response to 0.49 ± 0.15 (n = 2). Accordingly, all other experiments were performed in extracellular solutions containing 2 mM calcium (see *Materials and Methods*). Receptors formed from human $\alpha4\beta2$ subunits also show this bell-shaped dependence on external calcium ion concentration (Buisson et al., 1996), in contrast to some other types of neuronal nicotinic receptors (Mulle et al., 1992; Vernino et al., 1992). Buisson et al. (1996) have shown that the reduction in response at high external calcium ion concentrations reflects a reduced single channel conductance.

Physostigmine Potentiates Responses to Low Concentrations of Acetylcholine or Nicotine. It has been reported that the anticholinesterase, physostigmine, can act as a weak agonist at some nicotinic receptors (Pereira et al., 1994) and that 1-methyl-galanthamine can potentiate the response of PC12 cells to low concentrations of acetylcholine (Storch et al., 1995). We found that physostigmine (1 μM) can potentiate responses to both acetylcholine and nicotine. Responses to 1 μM nicotine were potentiated by about 2-fold in

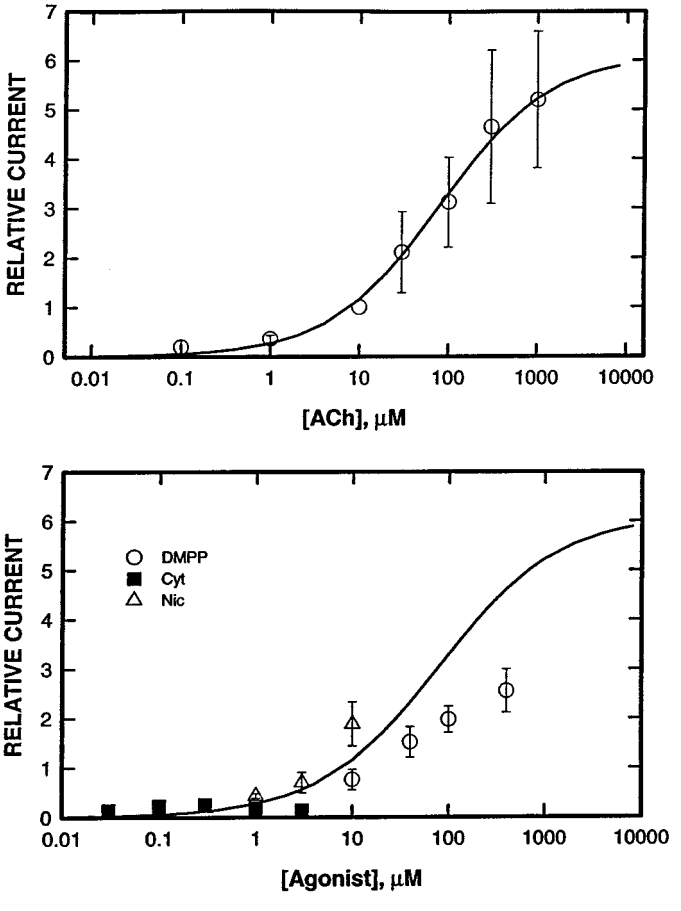

Fig. 5. Concentration-response relationship for activation of currents. The upper panel shows responses to applications of acetylcholine, normalized to the response in the same cell to 10 μM acetylcholine. The solid line shows the fit of eq. 1 to the data (K_h 80 μM, N_h 0.7, maximal relative response 6.1). The lower panel shows responses to other agonists, again normalized to the response to 10 μM acetylcholine (abbreviations: NIC, nicotine; DMPP, dimethylphenylpiperazinium; CYT, cytisine). The solid line repeats the curve from the upper panel to summarize the concentration-response relationship for acetylcholine. Symbols show the means of 3 to 17 values (±1 S.D., when larger than the symbol size).

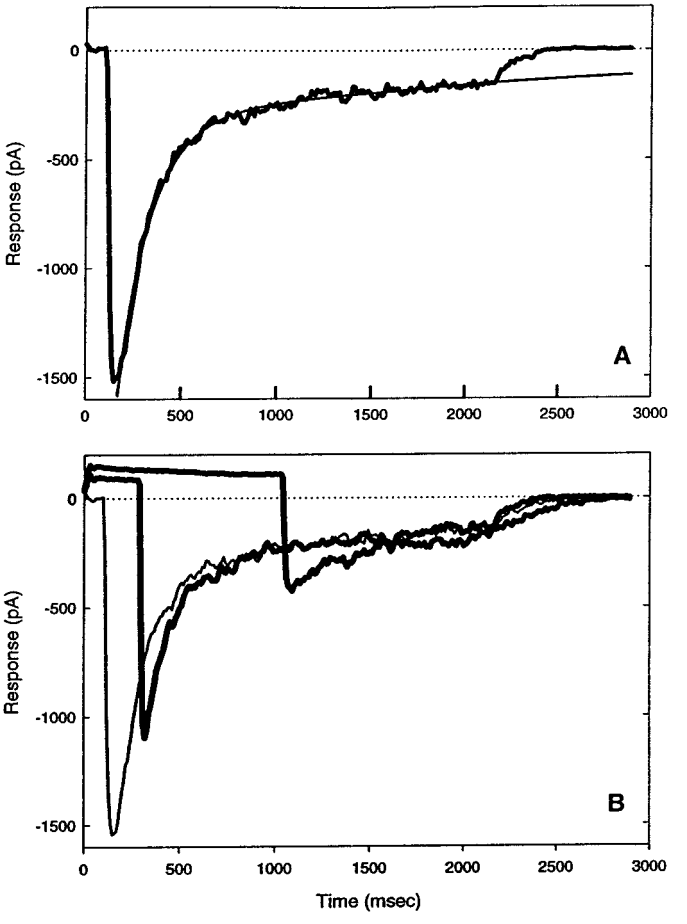

Fig. 6. Desensitization to prolonged applications of 100 μM acetylcholine. The upper panel shows a response to a 2-s application of 100 μM acetylcholine, made to a cell held at −100 mV. Overlaid on the data trace is the sum of two exponentials, declining to a baseline of zero (that is, it was assumed that eventually the desensitization would have been complete). The fitted values are: fast decaying component time constant 170 ms, slow component time constant 2700 ms, and fraction fast 0.8. Overall, immediately after breaking into the cell, the values were: fast component time constant 183 ± 52 ms, slow component time constant 2040 ± 720 ms, and fraction fast 0.7 ± 0.08 (n = 10). The lower panel shows three superimposed responses. In one (thin line), the cell was held at −100 mV throughout the response. In the others (thicker lines), the cell was held at +50 mV for the initial 250 ms or 1000 ms of the application of 100 μM acetylcholine. The responses on jumping to −100 mV are clearly larger than the control response; when the cell was held at +50 mV for 250 ms, the response immediately on jumping to −100 mV was about 1.4 times control, whereas when the cell was held at +50 for 1000 ms, it was about 1.9. Overall, after 250 ms the response was increased to 1.5 ± 0.3 times control (n = 6), whereas after 1000 ms it was 1.8 ± 0.3 (n = 15).

the presence of 1 μM physostigmine (2.1 $\pm$ 0.9; n = 5). The effect on responses to acetylcholine depended on the concentration of acetylcholine used. Responses to 0.1 μM acetylcholine were potentiated by 1.8-fold ($\pm$0.4, n = 3), those to 1 μM acetylcholine by 1.2-fold (1.2 $\pm$ 0.2, n = 10) and those to 10 μM acetylcholine by only 1.1-fold ($\pm$0.03, n = 3).

It has been reported that codeine has similar properties to physostigmine in the ability to activate neuronal nicotinic receptors (Storch et al., 1995). In HN42 cells, 0.4 μM codeine had no effect on responses to 1 μM acetylcholine (responses were 0.9 $\pm$ 0.2-fold in the presence of codeine, n = 5) and to 10 μM acetylcholine (0.9 $\pm$ 0.02; n = 2).

Inhibition of Responses by dHβE. dHβE reduced responses activated by nicotine or acetylcholine. The blocking action of dHβE was relatively slow to develop; when dHβE was applied simultaneously with an agonist, there was relatively little block. We studied the onset of block using 10 nM dHβE preapplied in the absence of agonist (Fig. 8), and found that the onset had a time constant of about 3 s (2.5 $\pm$ 0.8 s), with a final level of about 80% block (80 $\pm$ 2%, n = 4). A similar value of block after 30-s application of 10 nM dHβE was found in tests with 10 nM dHβE plus 1 μM nicotine (78 $\pm$ 9%, n = 2). If we assume that the block of response can be described by a simple binding isotherm and that the concentration of agonist used is low compared with the affinity, then this would correspond to an apparent affinity for dHβE of about 2.5 nM (given 80% block at 10 nM). We then examined the dissociation rate of dHβE by applying a long (10–30 s) pulse of a high concentration (100 nM) of dHβE to fully block the response, and then washing for different periods before testing with agonist (Fig. 8). When the recovery data were fit with single exponentials, the mean time constant was about 9 s (9.4 $\pm$ 5.4 s, n = 11). The data from the onset time course predict a time constant for recovery of about 13 s, assuming that the block is described by a simple binding equilibrium (because the onset rate would be sum of the blocking and unblocking rates). dHβE blocked receptor activation by agonists somewhat more potently than it inhibited agonist binding to cell homogenates. This might be the result of the different experimental conditions, or might indicate that dHβE binds to desensitized receptors with a lower affinity.

In preliminary experiments, 100 nM methyllycaconitine blocked responses by 95% (10 μM acetylcholine, n = 2). The neuromuscular blocking agents *d*-tubocurarine and pancuronium were relatively ineffective; 10 μM *d*-tubocurarine blocked responses to 1 μM acetylcholine by about 30% (33 $\pm$ 8%, n = 5) while 1 μM pancuronium had no effect (n = 5).

Inhibition of Responses by +ACN. It has been reported that progesterone inhibits recombinant chicken $\alpha4\beta2$ receptors (Valera et al., 1992) and that anesthetics of various classes inhibit receptors formed from rat (Violet et al., 1997) or chicken (Flood et al., 1997) $\alpha4\beta2$ subunits expressed in *Xenopus* oocytes. Accordingly, we examined the ability of an anesthetic neuroactive steroid to inhibit responses of these cells. Cells were pre-exposed to +ACN for 30 s before acetylcholine was applied, because preliminary results indicated that the steroids had little effect when applied simultaneously with acetylcholine. As summarized in Fig. 4 (open

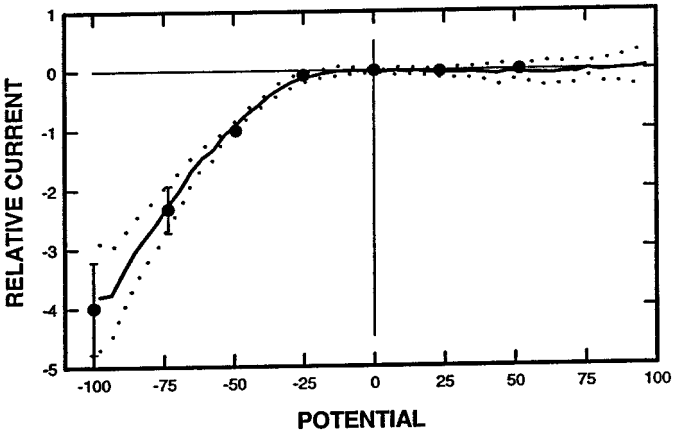

Fig. 7. Current-voltage relationship for nicotinic responses. Acetylcholine was applied to cells held at potentials between $-$100 mV and +50 mV (symbols; seven or eight cells), or alternatively the membrane potential was changed by a linear ramp between $-$100 mV and +100 mV (solid line shows mean for data from 12 cells, dotted lines show $\pm$ 1 S.D.). Note that there is no indication of outward current even at potentials between +50 and +100 mV. The amplitudes are normalized to the response at $-$50 mV.

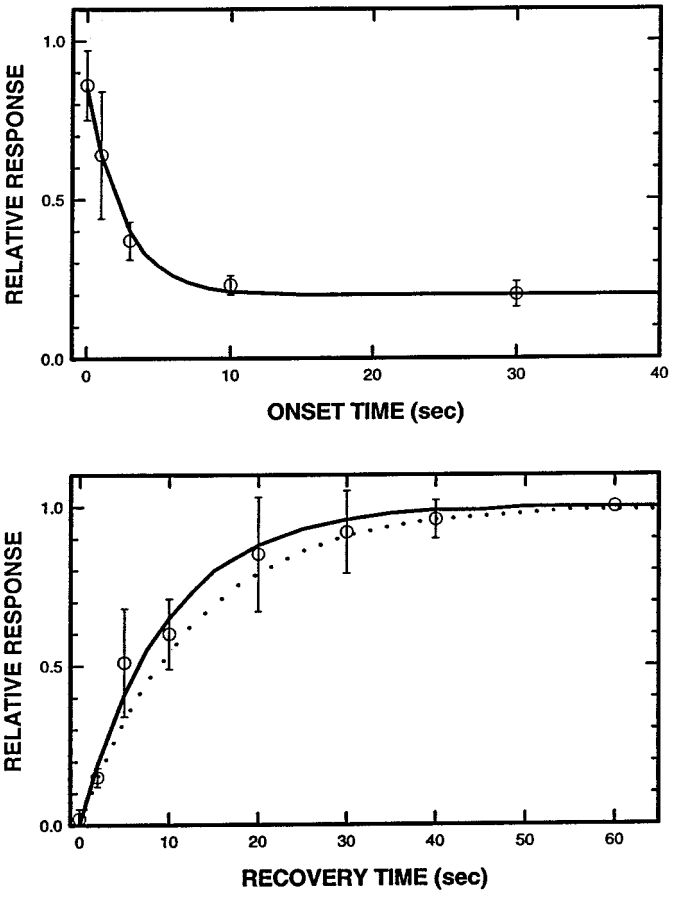

Fig. 8. Block of responses by dHβE. The upper panel shows the onset of the block of acetylcholine responses by 10 nM dHβE (4 cells). dHβE was applied for the time shown and then the application was switched to 10 μM acetylcholine plus 10 nM dHβE to assay the response. The solid line shows a single exponential decay with the mean time constant and equilibrium block (time constant 2.5 $\pm$ 0.4 s, final response 0.20 $\pm$ 0.02; four cells). The lower panel shows the recovery of the response after complete block by a 10-s exposure to 100 nM dHβE. The response was tested with either 1 μM acetylcholine (five cells) or 1 μM nicotine (six cells). The solid line shows an exponential recovery curve with the mean time constant from the data (time constant 9.4 $\pm$ 5.4 s, n = 11). The dotted line shows a recovery curve with the time constant predicted from the onset data (time constant 12.7 s). Note that zero time is offset in both panels.

circles), +ACN blocked responses to 1 μM acetylcholine with an IC_{50} of about 2 μM and a Hill coefficient of about 2. Because these concentrations of +ACN do not affect cytisine binding (Fig. 4), it appears that the mechanism of inhibition is not competitive. Similarly, the value of the Hill coefficient suggests that the mechanism involves the binding of more than one +ACN molecule to a receptor. DMSO (0.3%; 42 mM) did not affect the amplitude of responses to acetylcholine (relative response 0.99 $\pm$ 0.04; n = 3).

Discussion

The results indicate that there is a single class of high-affinity sites for cytisine and nicotine in the stably transfected cells, and that both nicotine and cytisine bind to the same site. dHβE behaves as a competitive inhibitor for binding of cytisine. dHβE also inhibits currents activated by acetylcholine and nicotine at similar concentrations, suggesting that the sites found in equilibrium binding experiments are the same agonist binding sites involved in receptor activation.

The affinities of cytisine and nicotine are comparable to the highest affinity values obtained in previous studies on homogenates from rat brain or spinal cord, whereas the specific activity is about 4-fold higher (Table 1). The affinity for dHβE also is comparable to the highest values reported previously (Table 1). The higher apparent affinities we observe may arise from the existence of a homogeneous population of binding sites.

Our studies of receptor activation suggest that nicotine and acetylcholine are equally potent and effective at activating receptors, whereas dimethylphenylpiperazinium appears to be less potent and less effective, and cytisine may be more potent but has a smaller maximal effect. These observations are broadly consistent with the results of previous studies of rat $\alpha4\beta2$ receptors expressed in Xenopus oocytes (Luetje and Patrick, 1991; Fenster et al., 1997). The apparent EC_{50} for activation by acetylcholine is about 80 μM (previous values range from 3–100 μM) and the apparent Hill slope is about 0.7 (previous values range from 0.8–1.1) (Papke and Heinemann, 1994; Stafford et al., 1994; Violet et al., 1997). However, the shape of the concentration-response curves may have been affected by desensitization. In addition, our data with cytisine or dimethylphenylpiperazinium may be affected by channel blocking activity by these agonists. Hence, full characterization of the activation of these receptors will require further experimentation.

Human $\alpha4\beta2$ receptors have also been stably expressed in HEK293 cells (K177 cells; Gopalakrishnan et al., 1996). The specific binding of cytisine to homogenates prepared from K177 cells is about 1400 fmol/mg protein (3-fold higher than we observed), and the affinities for cytisine (0.2 nM), nicotine (1.1 nM) and dHβE (60 nM) are similar (Gopalakrishnan et al., 1996). However, in functional studies, the EC_{50} for activation by acetylcholine is about 3 μM and the Hill slope 1.2 (Buisson et al., 1996), showing half-maximal activation at significantly lower acetylcholine concentrations than in HN42 cells. In K177 cells, nicotine is slightly more potent (EC_{50} 1.2 μM) and effective (maximal response about 1.2 times the maximal response to acetylcholine) than is acetylcholine, whereas cytisine is both less potent and less effective (Buisson et al., 1996). All of the agonists tested showed lower EC_{50} values with the human $\alpha4\beta2$ receptors than our data with rat $\alpha4\beta2$ receptors. This suggests functional differences between the two types of receptor, but additional work with the rat $\alpha4\beta2$ receptors is required before definite conclusions are drawn. In studies of K177 cells, dHβE has an IC_{50} against responses to 1 μM acetylcholine of about 80 nM and methyllycaconitine an IC_{50} of about 1.5 μM. Both of these values are higher than we found, possibly because the concentration of agonist which we used was further below the EC_{50} for activation of current. However, the fact that the apparent K_i for inhibition of cytisine binding by dHβE is also higher for K177 cells suggests that there might be species differences.

Responses to high concentrations of acetylcholine desensitized slightly more slowly when cells were held at +50 mV than at −100 mV. This contrasts with a previous study of chicken α4-nα1 subunits expressed in *Xenopus* oocytes, which showed no desensitization at positive membrane potentials (Gross et al., 1991). It is possible that the difference arises from the expression system used. However, the amount of desensitization we observed at +50 mV is also much greater than that reported for rat sympathetic ganglion neurons, which show no desensitization at positive potentials (Mathie et al., 1990). Hence, there are clear differences among subunit combinations in this respect, and are likely to be differences among species as well.

Previous workers have examined the actions of the anticholinesterase agent, physostigmine, on neuronal nicotinic receptors (Pereira et al., 1993, 1994; Storch et al., 1995). Physostigmine, methyl-galanthamine and codeine have been reported to act as weak agonists in activating single channel

TABLE 1
Binding and displacement parameters for rat preparations

	Direct Binding				Displacement				
	Nicotine		Cytisine						
	B_{max}	K	B_{max}	K	Ligand	Cytisine K_i	Nicotine K_i	dHβE K_i	Ref.[a]
	fmol/mg	*nM*	*fmol/mg*	*nM*		*nM*	*nM*	*nM*	
Rat brain (P2)	115	0.9	99	0.20	Cytisine	0.14	0.7	17	1
Rat cortex	–	–	4	0.90	Cytisine	0.45	3.3	22	2
Rat cortex	57	3.5	–	–	Nicotine	2.00	4.2	91	3
Rat spinal cord	20	2.2	20	0.40	Cytisine	0.71	2.3	21	4
Rat mean		2.2		0.50		0.83	2.6	38	
(S.D.)		(1.1)		(0.29)		(0.71)	(1.3)	(31)	
HN42 cells	544	0.40	383	0.12	Cytisine	–	0.43	22	
(S.D.)	(117)	(0.01)	(73)	(0.05)				(2)	

[a] The references are: 1, Anderson and Arneric, 1994; 2, Pabreza et al., 1991; 3, Martino-Barrows and Kellar, 1987; 4, Khan et al., 1994.

openings. Activation is not blocked by drugs such as dHβE, but can be blocked by a monoclonal antibody which does not prevent binding of α-neurotoxin to the muscle nicotinic receptor (FK1). Methyl-galanthamine has also been reported to potentiate the whole-cell responses elicited by acetylcholine applied to PC12 pheochromocytoma cells (Storch et al., 1995). Based on these observations, it has been proposed that physostigmine and some other compounds can activate neuronal nicotinic receptors by binding to a site distinct from the identified acetylcholine-binding sites (Pereira et al., 1993, 1994; Storch et al., 1995). We found that physostigmine potentiates the responses of HN42 cells to low concentrations of nicotine or acetylcholine. Potentiation of nicotine responses indicates that antiesterase activity does not underlie the action. Our results do not directly address the mechanism of potentiation. However, other workers have found that physostigmine is ineffective at preventing cytisine binding to rat brain homogenates (Pabreza et al., 1991; Anderson and Arneric, 1994), which indicates that physostigmine does not interact strongly with the acetylcholine binding site. Although we confirmed a potentiating action of physostigmine on rat α4β2 type receptors, we found that codeine showed no potentiation on these receptors. This may be a result of different experimental approaches (potentiation versus activation) or different receptor subtypes in the cells.

Neuroactive steroids have been shown to potentiate responses to low concentrations of γ-aminobutyric acid (GABA) and to directly gate GABA-A receptors (reviewed in Paul and Purdy, 1992). +ACN is a potent anesthetic in tadpoles and mice that both potentiates and activates responses of GABA-A receptors (Wittmer et al., 1996). Potentiation of responses of cultured hippocampal neurons to 2 μM GABA has an EC_{50} of 1.4 μM, and direct gating of responses has an EC_{50} of 5 μM (Wittmer et al., 1996). We find, in contrast, that +ACN inhibits responses of the α4β2 neuronal nicotinic receptors with an IC_{50} of about 1.3 μM. +ACN is not a competitive antagonist for rat α4β2 nicotinic receptors because it has no effect on cytisine binding. The concentration dependence of inhibition indicates that there are at least two binding sites on each receptor. We are presently examining the structural requirements for this action of steroid. Other general anesthetic agents, including halogenated volatile agents and the i.v. agent propofol, also inhibit responses of receptors formed from rat (Violet et al., 1997) or chicken (Flood et al., 1997) α4β2 subunits. It is perhaps not surprising that these agents affect two members of the ligand-gated ion channel family, although it is interesting that they consistently have opposite actions on GABA-A and neuronal nicotinic α4β2 receptors. However, not all nicotinic receptors are equally affected; responses of neuronal nicotinic α7 (Flood et al., 1997) or muscle nicotinic (Violet et al., 1997) receptors are 10-fold
or more less sensitive to the anesthetics tested. Studies of the actions of additional drugs may reveal differences in the binding sites for anesthetics, as well as differences in the functional consequences of binding, for the GABA-A and nicotinic α4β2 receptors.

In summary, HN42 cells express a single class of high-affinity nicotine-binding sites. The binding properties are those expected from previous studies. Studies of the pharmacological properties of these receptors show that they can be positively or negatively modulated through allosteric sites, which may be important in understanding the role of α4β2 receptors in the brain.

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