Rat α3/β4 Subtype of Neuronal Nicotinic Acetylcholine Receptor Stably Expressed in a Transfected Cell Line: Pharmacology of Ligand Binding and Function

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ABSTRACT

We stably transfected human kidney embryonic 293 cells with the rat neuronal nicotinic acetylcholine receptor (nAChR) α3 and β4 subunit genes. This new cell line, KXα3β4R2, expresses a high level of the α3/β4 receptor subtype, which binds (±)-[3H]epibatidine with a Kd value of 304 ± 16 nM and a Bmax value of 8942 ± 115 fmol/mg protein. Comparison of nicotinic drugs in competing for the α3/β4 receptor binding sites in this cell line and the binding sites in rat forebrain (predominantly α4/β2 receptors) revealed marked differences in their Kd values, but similar rank orders of potency for agonists were observed, with the exception of anatoxin-A. The affinity of the competitive antagonist dihydro-β-erythroidine is >7000 times higher at α4/β2 receptors in rat forebrain than at the α3/β4 receptors in these cells. The α3/β4 nAChRs expressed in this cell line are functional, and in response to nicotinic agonists, 86Rb+ efflux was increased to levels 8–10 times the basal levels. Acetylcholine, (−)-nicotine, cytisine, carbamol, and (±)-epibatidine all stimulated 86Rb+ efflux, which was blocked by mecamylamine. The EC50 values for acetylcholine and (−)-nicotine to stimulate 86Rb+ efflux were 114 ± 24 and 28 ± 4 μM, respectively. The rank order of potency of nicotinic antagonists in blocking the function of this α3/β4 receptor was mecamylamine > d-tubocurarine > dihydro-β-erythroidine > hexamethonium. Mecamylamine, d-tubocarurine, and hexamethonium blocked the function by a noncompetitive mechanism, whereas dihydro-β-erythroidine blocked the function competitively. The KXα3β4R2 cell line should prove to be a very useful model for studying this subtype of nAChRs.

Neuronal nAChRs are ligand-gated cation channels composed of α and β subunits. Eight different α subunits (α2–α9) and three different β subunits (β2–β4) have been identified in vertebrate neuronal tissue, which allows for the possibility of multiple nAChR subtypes that display a range of functional and pharmacological characteristics. In rat brain, the two most abundant nAChR subtypes (as measured by the density of binding sites) are one composed of α4 and β2 subunits (Whiting et al., 1991; Flores et al., 1992) and another that may be composed of α7 subunits only (Schoepfer et al., 1990; Seguela et al., 1993). The relative abundance of the mRNA encoding nAChR subunits in brain is consistent with the prevalence of these two receptor subtypes (Wada et al., 1989; Seguela et al., 1993). However, the CNS expresses mRNA for all of the nAChR subunits, and despite their lower abundance in brain overall, the receptors that contain α3 subunits may mediate important effects of acetylcholine and nicotine in specific regions of the brain (Mulle et al., 1991; Connolly et al., 1995; Clarke and Reuben, 1996; Albuquerque et al., 1997; Kulak et al., 1997), in the retina (McKay et al., 1994), and the spinal cord (Flores et al., 1997).

In addition to their roles in the CNS, nAChRs comprised of α3 subunits in combination with either β2 or β4 subunits may play fundamental roles in peripheral neuronal tissues, including neurons of mammalian sympathetic ganglia (Coverton et al., 1994; Mandelzys et al., 1994), parasympathetic ganglia (Poth et al., 1997), sensory neurons such as the trigeminal ganglia (Flores et al., 1996), and adrenal chromaffin cells (Rogers et al., 1992; Campos-Caro, 1997). Nicotinic receptors containing α3 subunits also may predominate in chick ciliary ganglia (Boyd et al., 1988; Couturier et al., 1990), superior cervical ganglia (Couturier et al., 1990; Vennall et al., 1993; Conroy and Berg, 1995), and dorsal root ganglion cells (Boyd et al., 1991).

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; CNS, central nervous system; EB, (±)-epibatidine; DHβE, dihydro-β-erythroidine; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; [Ca2+], intracellular Ca2+ concentration; [Na+], intracellular Na+ concentration; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid.
Compared with the α4/β2 nAChR in the CNS, receptors that contain α3 subunits seem to have much lower affinity for most nicotinic agonists; therefore, until recently, they could not be reliably measured or studied with the available radiolabeled ligands. This fundamental obstacle has hindered studies of the pharmacology and regulation of the binding sites of these receptor subtypes. EB, in contrast to most other nicotinic agonists, has high affinity for ganglionic-type receptors (Badio and Daly, 1994); in fact, [3H]EB binds with high affinity to several different defined nAChR subtypes in stably transfected cell lines (Xiao et al., 1996), as well as to the receptor or receptors in rat adrenal gland (Houghtling et al., 1995) and trigeminal ganglia (Flores et al., 1996). Furthermore, the iodinated analog of EB, [125I]EB (formerly [125I]IPH), labels receptors in the superior cervical ganglia (Dávila-García et al., 1997). These ganglionic tissues seem to contain nicotinic receptors composed predominantly of α3 subunits in combination with either β2 or β4 subunits, and in some cases, there may be an additional α5 subunit (Conroy et al., 1992; Conroy and Berg, 1995).

Another critical obstacle to the characterization of the pharmacology and regulation of nAChR subtypes containing α3 subunits is that the tissues in which they are found in high concentration, such as autonomic ganglia, adrenal gland, and specific nuclei within the brain, may contain more than one subtype of nicotinic receptor, making the assignment of characteristics to any one subtype difficult. Furthermore, these tissues provide only a very limited amount of tissue for study because of their relatively small size.

To study the pharmacological and functional characteristics of an important nAChR subtype that contains α3 subunits, we transfected HEK 293 cells with the genes encoding rat α3 and β4 nicotinic receptor subunits. A stable clonal cell line has been established, and it expresses this nAChR subtype at a very high density. Here, we report the characteristics of the α3/β4 nAChR binding site labeled by [3H]EB and the activation of this receptor ligand-gated ion channel by nicotinic agonists.

**Experimental Procedures**

**Materials and drugs.** Tissue culture medium, serum, antibiotics, restriction endonucleases, modifying enzymes, and molecular size standards were obtained from Gibco BRL (Gaithersburg, MD). [3H]EB and 48RbCl were supplied by New England Nuclear Research Products (Boston, MA). [α-32P]dATPs, [α-32P]CTP, and [γ-32P]ATP were obtained from Amersham (Arlington Heights, IL). DNA sequencing reagents were purchased from United States Biochemicals (Cleveland, OH). Electrophoresis reagents were purchased from BioRad Laboratories (Melville, NY). All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated. A-85380, (+)-anatoxin-A, DHβE, and EB were from Research Biochemicals International (Natick, MA). (+)-Nicotine, acetylcholine, carbadox, d-tubocurarine, and cytisine were from Sigma Chemical. Mecamylamine was from Merck Sharp & Dohme Research Lab (Rahway, NJ). Two plasmids, pPCA48E-3 and pZPC13, which carry the cDNA clones of rat neuronal nAChR subunit α3 and β4 genes, were described previously (Boulter et al., 1987; Duvoisin et al., 1989) and were generously provided by Dr. J. Boulter (Salk Institute, La Jolla, CA). HEK 293 cells (CRL 1573; American Type Culture Collection, Rockville, MD) were a gift from Y.-H. Wang and B.B. Wolfe (George town University, Washington, D.C.).

**Construction of pKXα3RC1 and pKXβ4RC1.** To constitutively express the rat neuronal nAChR α3 subunit in mammalian cells, a 1.7-kb HindIII/EcoRI fragment was isolated from the pPC44E8E-3 and subcloned into the eukaryotic expression vector pcDNA3 (In Vitrogen, San Diego, CA). The resulting plasmid with the α3 coding sequence in the sense orientation was designated pKXα3RC1. For generation of the eukaryotic expressible β4 gene, a 2-kb EcoRI/XbaI fragment was isolated from the pZPC13 and inserted into the pcDNA3 vector. The new construct with the β4 coding sequence in the sense orientation was denoted pKXβ4RC1. pKXα3RC1 and pKXβ4RC1 were restriction mapped and sequenced to confirm correct ligation and orientation.

**Cell culture and stable transfection.** HEK 293 cells were maintained at 37°C with 5% CO₂ in a humidified incubator. Growth medium for the HEK 293 cells was minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. For transfection experiments, plasmids were linearized by restriction digestion within their prokaryotic elements. Transfection was conducted according to the calcium phosphate method (Chen and Okayama, 1987). Briefly, exponentially growing HEK 293 cells were plated onto 100-mm dishes containing 10 ml of the growth medium 24 hr before transfection. For a single transfection experiment, 1 ml of transfection mixture was made. The mixture was composed of 10 μg of linearized DNA, 125 mM CaCl₂, 25 mM HEPES, 140 mM KCl, 6 mM glucose, and 0.75 mM Na₃HPO₄, pH 7.05. The mixture was added to the dish of cells in dropwise fashion. The cells were incubated with the transfection mixture for 16 hr in the incubator, after which the mixture was removed, and the cells were grown in fresh growth medium for 24 hr. The cells then were collected and plated at a range of densities onto six-well plates in the selection medium, which consisted of the growth medium containing 0.7 mg/ml geneticin (G418). The cells were grown in the selection medium for 3–4 weeks before G418-resistant clones were picked up by cloning cylinders. The stably transfected cells were maintained in the selection growth medium.

**DNA isolation and RNase protection assay.** Total cellular RNA was isolated by using RNA-STAT-60, an RNA isolation reagent (Tel Test B, Friendswood, TX). DNA templates for antisense riboprobes were prepared as described previously (Xiao et al., 1995). The size of the full-length probes and the expected protected fragments of the probes are for rat α2, 421 and 332 bases; rat α3, 308 and 231 bases; rat α4, 497 and 408 bases; rat α5, 450 and 388 bases; rat β2, 328 and 266 bases; rat β4, 258 and 174 bases; and human GAPDH, 224 and 154 bases, respectively. The probes were synthesized using T7 RNA polymerase (Ambion, Austin, TX) and [α-32P]CTP. Specific activities of [α-32P]CTP used for synthesizing the probes of rat nAChR subunit genes and the probe of GAPDH were 800 and 32 Ci/mmol, respectively. Approximately 50 μg of total RNA was hybridized with probes overnight at 42°C. Nonprotected probes were digested with a mixture of RNase A and RNase T₁, and the samples were processed using the RPA II kit (Ambion). The sizes of protected fragments were determined by electrophoresis on a 6% denaturing polyacrylamide gel.

**Ligand binding studies.** Cultured cells at ~70% confluence were harvested at 50 mm Tris-HCl, pH 7.4, and homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged at 35,000 × g for 10 min, and pellets were washed twice with fresh buffer. Membrane pellets were resuspended in fresh buffer, and aliquots equivalent to 60–200 μg of protein were used for binding assays. Ligand binding was measured as described previously (Houghtling et al., 1995) with minor modifications. Briefly, membrane preparations were incubated with [3H]EB for 4 hr at 24°C in a final volume of 2.5 ml. Bound and free ligands were separated by vacuum filtration through Whatman GF/C filters treated with 0.5% polyethyleneimine. The filter-retained radioactivity was determined by liquid scintillation counting. Total binding and nonspecific binding was determined in the absence and presence of (−)-nicotine (300 μM), respectively. Specific binding was defined as the difference between total binding and nonspecific binding. Typically, total binding was measured in duplicate, and nonspe-
pecific binding was measured in singlet. In saturation binding experiments, receptor densities ($B_{\text{max}}$) and dissociation constants ($K_d$) were determined by nonlinear least-squares regression analyses (Accuit Saturation Two-Site Program; Beckman Instruments, Fullerton, CA). The one-site model was accepted unless the two-site model gave a statistically better fit of the data ($p < 0.05$ by F test). Hill coefficients ($n_H$) of specific binding curves were determined by linear regression analyses of those specific binding values that fell between 10% and 90% of $B_{\text{max}}$. The affinities of drugs at the receptors were determined from binding inhibition curves, in which a series of concentrations of each drug was incubated with a single concentration of $[^{3}H]$EB. In some studies, the type of binding inhibition mechanism was investigated by including the drug in a $[^{3}H]$EB binding saturation assay. Hill coefficients ($n_H$) and IC$_{50}$ values of binding inhibition curves were determined by linear regression analyses. The inhibition constants ($K_i$) were calculated according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

To determine the fraction of nAChRs on the cell surface, $[^{3}H]$EB binding was measured in intact cells according to a method that was adapted from one used for other receptors (Koenig and Edwardson, 1997) with minor modifications. Briefly, cells were plated onto 24-well plates coated with poly-d-lysine and grown in selection medium for 14–20 hr to reach ~30% confluency. The medium was removed and replaced by 20 mM HEPES buffer, pH 7.4, containing minimum essential medium and 3 nM $[^{3}H]$EB. The intact cells were incubated with $[^{3}H]$EB for 2 hr, after which the buffer was removed by aspiration and the cells were washed rapidly three times with buffer. The cells then were lysed by the addition of 1 ml of 0.1 M NaOH, and the lysate in each well was counted in a liquid scintillation counter.

$[^{32}Rb]^{86}$ efflux assay. The function of nAChRs expressed in the transfected cells was measured using a $[^{32}Rb]^{86}$ efflux assay as described by Lukas and Cullen (1988), with modifications. In brief, identical aliquots of cells in the selection growth medium were plated onto 24-well plates coated with poly-d-lysine. The plated cells were grown at 37° for 18–24 hr to reach 70–85% confluence. The cells then were incubated in growth medium (0.5–1 ml/well) containing $[^{32}Rb]^{86}$Cl$^-$ (2 μCi/ml) for 4 hr at 37°. The loading mixture was aspirated, and the cells were washed three times with 1 ml aliquots of buffer (15 mM HEPES, 140 mM NaCl, 2 mM KCl, 1 mM MgSO$_4$, 1.8 mM CaCl$_2$, 11 mM glucose, pH 7.4) for 30 sec, 5 min, and 30 sec, respectively. One milliliter of buffer, with or without drugs, was added to each well. After incubation for 2 min, the assay buffer was collected, and the amount of $[^{32}Rb]^{86}$ in the buffer was determined. Cells were lysed by the addition of 1 ml of 0.1 M NaOH to each well, and the lysate was collected for determination of the amount of $[^{32}Rb]^{86}$ that was in the experiments.

**Fig. 1.** Multiple-probe RNase protection assay of expression of nAChR subunit genes in HEK 293 cells and transfected cell lines. RNase protection assay was carried out as described in Experimental Procedures. Total RNA from each cell line was hybridized with a mixture of antisense probes labeled with $[^{32}P]$ for six rat nAChR subunit genes ($\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\beta_2$, $\beta_4$) and the human GAPDH gene. Nonprotected probes were digested with a mixture of RNase A and RNase T1. The sizes of protected RNA fragments were determined by electrophoresis on a 6% denaturing polyacrylamide gel. HEK 293, the parental cell line for transfection experiments; KXc1, the cell line that was transfected with the vector, pcDNA3; KXc3R1, the cell line that was transfected with rat $\alpha_3$ subunit gene; KXp4R1, the cell line that was transfected with rat $\beta_4$ subunit gene; KXa3B4R2, the cell line that was transfected with both rat $\alpha_3$ and $\beta_4$ subunit genes. Labels, specific protected fragments and their sizes (bases). The GAPDH was used as an internal and loading control in the experiments.

**Fig. 2.** Specific binding of $[^{3}H]$EB to membrane homogenates from HEK 293 cells, transfected cells, and rat forebrain (RFB). Binding assays were carried out as described in Experimental Procedures. The concentration of $[^{3}H]$EB used in these experiments was ~8 nM. Total binding and nonspecific binding was determined in the absence and presence of 300 μM (−)-nicotine, respectively. Specific binding was defined as the difference between total binding and nonspecific binding. See the legend for Fig. 1 for designations of cell lines. Values are the mean ± standard error from three to six independent experiments.
cells at the end of the efflux assay. Radioactivity of the assay samples and lysates was measured by liquid scintillation counting. Total loading (cpm) was calculated as the sum of the assay sample and the lysate of each well. Values of total loading were 100,000–200,000 cpm/well. The amount of $^{86}$Rb$^+$ efflux was expressed as a percentage of $^{86}$Rb$^+$ loaded. Stimulated $^{86}$Rb$^+$ efflux was defined as the difference between efflux in presence and absence of nicotinic agonists. The EC$_{50}$, I$_{C50}$, and n$_H$ values were estimated by linear regression analysis. Experiments with antagonists were done in two different ways. For obtaining an IC$_{50}$ value, inhibition curves were constructed in which different concentrations of an antagonist were included in the assay to inhibit efflux stimulated by 100 µM nicotine. For determination of the mechanism of antagonist blockade, concentration-response curves for activation by nicotine were constructed in the absence or presence of one or more concentrations of an antagonist.

Ca$^{2+}$ and Na$^+$ imaging assays. Effects of nicotinic drugs on changes in [Ca$^{2+}$], or [Na$^+$], were evaluated by Ca$^{2+}$ and Na$^+$ imaging assays. These assays were performed on an Attofluor Fluorescence Imaging System (Attofluor, Rockville, MD) with an Axiovert 135 fluorescence microscope (Zeiss, Germany). Cells were plated onto 25-mm glass coverslips (Fisher, Pittsburgh, PA) or 35-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark), both precoated with poly-d-lysine. The plated cells were grown for 36–72 hr at 37°C. Culture medium was removed, and cells were rinsed three times with Locke’s buffer with the following composition: 140 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO$_3$, 1.3 mM CaCl$_2$, 1 mM MgCl$_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4.

For measuring [Ca$^{2+}$], cells were loaded with 5–10 µM Fura-2/AM (Molecular Probes, Eugene, OR) in Locke’s buffer supplemented with 0.1% Pluronic F-127 (Molecular Probes) to facilitate dye loading. The cells were incubated with the loading solution for 30 min at room temperature and then washed three times with Locke’s buffer. Dishes and coverslips were mounted onto the fluorescence microscope stage and then incubated with the Locke’s buffer for 15–20 min to complete Fura-2/AM deesterification. Buffer with or without drugs was delivered at a flow rate of 1 ml/min. Fluorescence was measured...
at room temperature at excitation wavelengths of 334 and 380 nm using dry objective 40/0.6 LD Achromplan for cells plated onto plastic dishes or oil immersion objective 40/1.3 Fluor Ph3 for cells seeded onto glass coverslips. Emission wavelengths, monitored with intensified CCD cameras, were >420 nm for objectives 40/0.6 LD Achromplan and >510 nm for objectives 40/1.3 Fluor Ph3. The [Ca\(^{2+}\)] was calculated from the ratio (R) of fluorescence signals obtained at the two excitation wavelengths as described by Grynkiewicz et al. (1985).

\( R_{\text{min}} \) values were measured when sodium ions were completely replaced by K\(^+\). \( R_{\text{max}} \) values were measured when sodium ions were completely replaced by K\(^+\) ions for room temperature correction.

Imaging of Na\(^+\) was carried out as described above for Ca\(^{2+}\) imaging with the following differences. Cells were loaded with 10 \( \mu \)M SBFI/AM (Molecular Probes) in Locke’s buffer supplemented with 10 mM EGTA and 10 \( \mu \)M ionomycin (Calbiochem, San Diego, CA) for 5–10 min in the absence of Ca\(^{2+}\). \( R_{\text{max}} \) values were obtained by subsequent incubation of the cells in Locke’s buffer supplemented with 10 mM CaCl\(_2\) and 10 \( \mu \)M ionomycin for 5–10 min. The background fluorescence then was measured in the presence of 10 mM MnCl\(_2\). The \( K_d \) value of the Fura-2/Ca\(^{2+}\) complex used for the calculation was 396 nm without correction for room temperature.

Stable transfections of HEK 293 cells. Four sets of plasmid DNA were used for transfection experiments independently: (1) pcDNA3 (vector only), (2) pKX\(_{a3RC1}\) (\( \alpha_3 \) subunit gene), (3) pKX\(_{b4RC1}\) (\( \beta_4 \) subunit gene), and (4) the combination of pKX\(_{a3RC1}\) and pKX\(_{b4RC1}\) (\( \alpha_3 \) and \( \beta_4 \) subunit genes). From each of the transfections, 36–72 stable, G418-resistant cell clones were isolated after cultivation in selection medium for 3–4 weeks. These clonal cell lines were then grown in selection medium for an additional 4 weeks.

Initial screening of the G418-resistant clonal cells was carried out by using multiple-probe RNase protection assays to measure mRNA in the total RNA preparation isolated from each clone. Stably transfected cell lines representing each of the transfection experiments were selected and designated as (1) KXC1 (transfected with the vector pcDNA3 only), (2) KX\(_{a3R1}\) (expressing the \( \alpha_3 \) subunit gene only), (3) KX\(_{b4R1}\) (expressing the \( \beta_4 \) subunit gene only), and (4) KX\(_{a3b4R2}\) (expressing both the \( \alpha_3 \) and \( \beta_4 \) subunit genes).

An assessment of the mRNA for six different rat nAChR subunits (\( \alpha_2 \sim \alpha_5, \beta_2, \) and \( \beta_4 \)) in the parent HEK 293 cells and in each of the four clonal cell lines is shown in Fig. 1. As expected, there was no detectable expression of mRNA for any of the six nAChR subunit genes in either HEK 293 or KXC1 cells. However, the mRNA levels for the appropriate subunit gene in KX\(_{a3R1}\) and KX\(_{b4R1}\) were quite high, as were the mRNA levels of both subunit genes in KX\(_{a3b4R2}\) (Fig. 1).

The clones that expressed the highest levels of the expected nAChR subunit mRNA or mRNAs were tested for their ability to bind \(^{3}H\)EB. At a \(^{3}H\)EB concentration of \(< 3 \text{ nmol/mL of protein} \) was detected in membrane homogenates from HEK 293 cells, from cells transfected with pcDNA3 only, or from cells transfected singly with either the \( \alpha_3 \) or \( \beta_4 \) subunit gene (Fig. 2). In contrast, in membrane homogenates from KX\(_{a3b4R2}\) cells, which had been transfected with both \( \alpha_3 \) and \( \beta_4 \) genes, specific binding of \(^{3}H\)EB was quite high, displaying a density of binding sites \( > 8000 \text{ fmol/mg of protein} \) (Fig. 2). As a point of comparison, the density of nicotinic receptors labeled by \(^{3}H\)EB in rat brain membrane homogenates (primarily \( \alpha_4/\beta_2 \) receptors) in parallel assays was \( \sim 88 \text{ fmol/mg protein} \), or \( \sim 1\% \) of the density of \( \alpha_3/\beta_4 \) receptors in this cell line (Fig. 2, inset). The KX\(_{a3b4R2}\) cell line was selected for further study and has been continually cultured in our laboratory for >12 months with no significant change in the levels of expression of the mRNA encoding \( \alpha_3 \) or \( \beta_4 \) subunits or in the binding to or function of the receptor.

### Results

#### Stable transfections of HEK 293 cells

Four sets of plasmid DNA were used for transfection experiments independently: (1) pcDNA3 (vector only), (2) pKX\(_{a3RC1}\) (\( \alpha_3 \) subunit gene), (3) pKX\(_{b4RC1}\) (\( \beta_4 \) subunit gene), and (4) the combination of pKX\(_{a3RC1}\) and pKX\(_{b4RC1}\) (\( \alpha_3 \) and \( \beta_4 \) subunit genes). From each of the transfections, 36–72 stable, G418-resistant cell clones were isolated after cultivation in selection medium for 3–4 weeks. These clonal cell lines were then grown in selection medium for an additional 4 weeks.

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### Analysis of \(^{3}H\)EB binding to \( \alpha_3/\beta_4 \) receptors in KX\(_{a3b4R2}\) cell membrane homogenates

Specific binding of \(^{3}H\)EB in cell membrane homogenates was saturable and represented \( > 98\% \) of the total binding throughout most of the concentration range used (Fig. 3A). The high density of nicotinic receptor binding sites in KX\(_{a3b4R2}\) cells and the very low nonspecific binding of \(^{3}H\)EB allowed binding to be analyzed over a wide range of concentrations. Over this range, \(^{3}H\)EB binding fit a model for a single site with a Hill

### TABLE 1

Comparison of pharmacological properties of nAChR ligand binding in membrane homogenates from KX\(_{a3b4R2}\) cell and rat forebrain

<table>
<thead>
<tr>
<th>Drug</th>
<th>KX(_{a3b4R2}) cell</th>
<th>Rat forebrain</th>
<th>Ratio (( K_d ) for KX(_{a3b4R2})/( K_d ) for rat forebrain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\pm))-Epibatidine</td>
<td>0.38 ± 0.07</td>
<td>0.053 ± 0.002</td>
<td>7</td>
</tr>
<tr>
<td>((\pm))-Anatoxin-A</td>
<td>52.9 ± 4.2</td>
<td>6.51 ± 0.53</td>
<td>8</td>
</tr>
<tr>
<td>A85380</td>
<td>73.6 ± 6.3</td>
<td>0.097 ± 0.023</td>
<td>759</td>
</tr>
<tr>
<td>Cytisine</td>
<td>195 ± 14</td>
<td>1.03 ± 0.14</td>
<td>189</td>
</tr>
<tr>
<td>((\pm))-Nicotine</td>
<td>475 ± 52</td>
<td>7.22 ± 0.23</td>
<td>66</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>881 ± 154</td>
<td>32.8 ± 10.5</td>
<td>27</td>
</tr>
<tr>
<td>Carbachol</td>
<td>3,839 ± 276</td>
<td>428 ± 57</td>
<td>9</td>
</tr>
<tr>
<td>d-Tubocurarine</td>
<td>22,929 ± 3,800</td>
<td>2,237 ± 1,911</td>
<td>10</td>
</tr>
<tr>
<td>DH(_2)E</td>
<td>218,622 ± 32,734</td>
<td>28.9 ± 15.2</td>
<td>7,565</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>&gt;1,000,000</td>
<td>102,864 ± 7,632</td>
<td>0.85 ± 0.02</td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>&gt;1,000,000</td>
<td></td>
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</tr>
</tbody>
</table>

\( ^a \) \( n_H \) significantly different from 1 (\( p < 0.05 \)).

\( ^b \) \( n_H \) significantly different from 1 (\( p < 0.001 \)).
coefficient \( (n_H) \) close to 1, a \( K_d \) value of \( \sim 300 \) pM, and a site density of \( \sim 8900 \) fmol/mg of protein (Fig. 3B, see figure legend for calculated values).

**Pharmacological characteristics of \( \alpha_3/\beta_4 \) receptor binding sites in KX\( \alpha_3 \beta_4 \)R2 cells.** The pharmacological characteristics of the \( \alpha_3/\beta_4 \) nicotinic receptor binding sites in these cells were examined in binding competition assays in which drugs competed against \( \sim 500 \) pM \([3H]EB \) for binding sites in cell membranes (Fig. 4A, Table 1). EB was by far the most potent drug in competing for \( \alpha_3/\beta_4 \) receptor binding sites, with an affinity >2 orders of magnitude higher than that of any other drug tested. For example, among nicotinic agonist drugs, EB was >100 times more potent than anatoxin-A and A85380, >500 times more potent than cytisine, and >1000 times more potent than nicotine, acetylcholine, or carbachol (Fig. 4, Table 1). Among nicotinic antagonists, \( d \)-tubocurarine was nearly 10 times more potent than DH\( \beta \)E in competing for \( \alpha_3/\beta_4 \) receptor binding sites (Fig. 4, Table 1).

**Fig. 5.** Effects of nicotinic agonists and antagonists on \( ^{86} \text{Rb} \) efflux from KX\( \alpha_3 \beta_4 \)R2 cells. \( ^{86} \text{Rb} \) efflux was measured as described in Experimental Procedures. Cells were loaded with \( ^{86} \text{Rb} \) and then exposed to buffer alone or buffer containing nicotinic drugs for 2 min. A, Effects of nicotine on \( ^{86} \text{Rb} \) efflux from HEK 293 cells and transfected cells. Cells were exposed to buffer alone (basal efflux) or buffer containing 100 \( \mu \)M nicotine. The amount of \( ^{86} \text{Rb} \) efflux was expressed as a percentage of \( ^{86} \text{Rb} \) loaded. Data shown are the mean \( \pm \) standard error from three separate measurements. B, Concentration-dependent activation of nAChR function in KX\( \alpha_3 \beta_4 \)R2 cells by nicotine and acetylcholine. The amount of \( ^{86} \text{Rb} \) efflux was expressed as a percentage of \( ^{86} \text{Rb} \) loaded. The stimulated \( ^{86} \text{Rb} \) efflux was defined as the difference between efflux with and without nicotinic agonists (i.e., basal efflux, normally 4–8%, has been subtracted). Data shown are representative of five independent experiments. Parameters were estimated by Hill plot analyses. The \( EC_{50} \) values (mean \( \pm \) standard error) for nicotine and acetylcholine were 28.2 \( \pm \) 3.7 and 113.7 \( \pm \) 24.2 \( \mu \)M, respectively. The Hill coefficients (mean \( \pm \) standard error) for nicotine and acetylcholine were 1.59 \( \pm \) 0.1 and 1.48 \( \pm \) 0.04, respectively. These Hill coefficients are significantly different from 1 \( (p < 0.01) \). The efficacy of acetylcholine relative to nicotine was 98.8 \( \pm \) 3\% (mean \( \pm \) standard error). C, Effects of nicotinic agonists on \( ^{86} \text{Rb} \) efflux from KX\( \alpha_3 \beta_4 \)R2 cells. Cells were exposed to buffer alone (basal efflux) or buffer containing nicotinic agonists, with or without mecamylamine. The amount of \( ^{86} \text{Rb} \) efflux was expressed as a percentage of \( ^{86} \text{Rb} \) loaded. The concentrations of drugs used were 30 \( \mu \)M \( (-) \)-nicotinic (Nic), 10 \( \mu \)M mecamylamine (Mec), 30 \( \mu \)M cytisine (Cyt), 300 \( \mu \)M carbachol (Carb), and 30 \( n \)M \( (+) \)-EB. Data shown are the mean \( \pm \) standard error from at least two independent measurements, each carried out in quadruplicate. D, Concentration-dependent inhibition by nicotinic antagonists of nicotine-stimulated \( ^{86} \text{Rb} \) efflux from KX\( \alpha_3 \beta_4 \)R2 cells. Cells were exposed to buffer alone or buffer containing 100 \( \mu \)M nicotine with or without antagonists. Control equaled the amount of \( ^{86} \text{Rb} \) efflux stimulated by 100 \( \mu \)M nicotine in the absence of antagonists. Parameters were estimated from Hill plots. The \( I_{50} \) and Hill coefficients were, respectively, 1.2 \( \pm \) 0.4 \( \mu \)M and 1.15 \( \pm \) 0.1 (three experiments) for mecamylamine, 9.6 \( \pm \) 1.4 \( \mu \)M and 0.78 \( \pm \) 0.03 (four experiments) for \( d \)-tubocurarine, 100 \( \pm \) 6 \( \mu \)M and 1.14 \( \pm \) 0.16 (three experiments) for DH\( \beta \)E, and 208 \( \pm \) 57 \( \mu \)M and 1.20 \( \pm \) 0.14 (five experiments) for hexamethonium (values are mean \( \pm \) standard error).
Neither mecamylamine nor hexamethonium at concentrations up to 1 mM competed effectively for \(^{3}H\)EB binding sites.

For comparison, the affinities of these same drugs were examined in parallel at nicotinic receptor binding sites labeled by \(^{3}H\)EB in rat forebrain membranes, which are predominantly the \(a3/b2\) subtype of nicotinic receptor (Whiting et al., 1991; Flores et al., 1992). All of the drugs examined were more potent in competing for the \(a3/b2\) receptor binding sites in rat forebrain than at the \(a3/b4\) receptors in KXa3b4R2 cells. This can be seen in the leftward shift of the curves in Fig. 4B compared with those in Fig. 4A. Furthermore, it should be noted that the affinity of \(^{3}H\)EB is \( \sim 7 \) times higher at the \(a4/b2\) receptors in rat forebrain than at the \(a3/b4\) receptors in KXa3b4R2 cells; therefore, the fraction of receptors occupied by \(^{3}H\)EB in rat forebrain was greater than that in the cells (90% versus 57%). Thus, as shown in Table 1, after accounting for this difference in receptor occupancy (Cheng and Prusoff, 1973), the differences in affinity of these drugs at the two receptor subtypes is actually greater than they appear from the curves in Fig. 4. The drugs that show the greatest differences in affinity at the two receptor subtypes are DH \(bE\) and A85380, which show, respectively, 7565 and 759 times higher affinity for \(a4/b2\) receptors than for \(a3/b4\) receptors. Cytisine, nicotine, and acetylcholine, all of which have been used as radioligands to label nicotinic receptors in brain, also have much higher affinity for \(a4/b2\) receptors than for \(a3/b4\) receptors (Table 1).

Among agonists, the rank order of binding affinities at the two receptor subtypes is similar, with only the relative position of anatoxin-A changing. In contrast, the order of affinities of the two antagonists, \(d\)-tubocurarine and DH \(bE\), is very different at the two receptor subtypes. Thus, at \(a3/b4\) receptors \(d\)-tubocurarine binds with almost 10 times higher affinity than DH \(bE\), whereas at \(a4/b2\) receptors, DH \(bE\) has 77 times higher affinity than \(d\)-tubocurarine (Table 1). Interestingly, the Hill coefficients for \(d\)-tubocurarine competing at the \(a3/b4\) receptor binding sites and for both antagonists competing at the binding sites in brain are significantly <1 (Table 1). Hexamethonium competed weakly for \(^{3}H\)EB binding sites in brain, whereas mecamylamine displayed virtually no affinity.

**Assessment of \(a3/b4\) receptor function in KXa3b4R2 cells.** To examine the function of the \(a3/b4\) nicotinic receptor in these cells, we measured ion flux stimulated by nicotinic agonists. Three types of ion flux were measured: efflux of \(^{86}\)Rb\(^{+}\) from preloaded cells, increases in intracellular Ca\(^{2+}\) as measured by Fura-2/Ca\(^{2+}\) imaging, and increases in intracellular Na\(^{+}\) as measured by SBFI/Na\(^{+}\) imaging.

**Stimulation of \(^{86}\)Rb\(^{+}\) efflux.** As shown in Fig. 5A, nicotine stimulated \(^{86}\)Rb\(^{+}\) efflux from KXa3b4R2 cells, which express both \(a3\) and \(b4\) subunits, but not from the parent HEK 293 cells or from HEK 293 cells transfected with either the vector only or the \(a3\) or \(b4\) subunit only. Both nicotine and acetylcholine stimulated \(^{86}\)Rb\(^{+}\) efflux from KXa3b4R2

cell lines in the presence of 2 mM mecamylamine. B, Effect of DH \(bE\). Control, concentration-response curve for activation by nicotine alone. DH \(bE\), concentration-response curve for activation by nicotine in the presence of 250 \(\mu\)M DH \(bE\). C, Effect of \(d\)-tubocurarine. Control, concentration-respose curve for activation by nicotine alone. \(d\)-TC, concentration-response curve for activation by nicotine in the presence of \(d\)-tubocurarine at the indicated concentrations.
cells in a concentration-dependent manner (Fig. 5B), with EC_{50} values of 28 and 114 μM, respectively. Maximal 86Rb⁺ efflux, 8–10 times greater than the basal efflux, occurred at a nicotine concentration of ~300 μM and an acetylcholine concentration of 2 mM. Concentrations of >1 mM nicotine and 10 mM acetylcholine tended to decrease 86Rb⁺ efflux, possibly due to desensitization or agonist blockade of the channel (see Fig. 6B). The Hill coefficients for functional activation of the receptor by nicotine and acetylcholine were 1.6 and 1.5, respectively, and in both cases, these values were significantly different from 1 (p < 0.01). In addition to nicotine and acetylcholine, cytisine (30 μM), carbachol (300 μM), and EB (30 nM) all stimulated 86Rb⁺ efflux from KXα3β4R2 cells, and in each case, the efflux was nearly completely blocked by 10 μM mecamylamine (Fig. 5C).

**Block of nicotine-stimulated 86Rb⁺ efflux.** The blockade of nicotine-stimulated 86Rb⁺ efflux by several antagonists was investigated. As shown in Fig. 5D, despite the inability of mecamylamine to compete for the agonist binding site (Table 1), it blocked nicotine-stimulated 86Rb⁺ efflux from KXα3β4R2 cells quite potently, with an IC_{50} value of ~1 μM and a nearly complete blockade of efflux at a concentration of 10 μM. Similarly, d-tubocurarine, DHβE, and hexamethonium blocked nicotine-stimulated ion efflux in a concentration-dependent manner, with IC_{50} values of ~10, 100, and 210 μM, respectively (Fig. 5D). In contrast to these drugs, α-bungarotoxin (1.5 μM) had no effect on nicotine-stimulated 86Rb⁺ efflux from KXα3β4R2 cells (data not shown).

Although Fig. 5D indicates the relative potency of these antagonists in blocking the α3/β4 receptor, it does not provide information about the type of block exerted by these drugs. Therefore, to investigate the mechanism of the block produced by these antagonists, concentration-response measurements for nicotine-stimulated 86Rb⁺ efflux were made in the absence and presence of each of the drugs. As shown in Fig. 6A, in the presence of mecamylamine or hexamethonium, the maximum response elicited by nicotine was decreased, but the nicotine EC_{50} value was not significantly altered, indicating that both of these drugs block these nicotinic receptors by a noncompetitive mechanism. In contrast, the presence of DHβE shifted the nicotine concentration-response curve to the right (Fig. 6B), resulting in a higher EC_{50} value but no decrease in the maximum response that could be elicited, indicating that DHβE blocks these receptors by a competitive mechanism.

Tubocurarine is a competitive antagonist at nicotinic receptors in muscle, whereas at ganglionic nicotinic receptors, it has been reported to block the ion channel of the receptor (Ascher et al., 1979), which would be consistent with a noncompetitive mechanism. Therefore, because d-tubocurarine might help to distinguish among nicotinic receptor subtypes, we investigated the type of mechanism by which it blocks α3/β4 receptor function. As shown in Fig. 6C, in the presence of increasing concentrations of d-tubocurarine, the maximum 86Rb⁺ efflux stimulated by nicotine was progressively decreased without noticeably affecting the nicotine EC_{50} value. Furthermore, this blockade by d-tubocurarine is reversible; therefore, when cells were exposed to 200 μM d-tubocurarine for 2 min (as in the 86Rb⁺ efflux assay) and then washed in fresh buffer, the response to nicotine was nearly completely restored (data not shown). Thus, d-tubocurarine blocks α3/β4 receptor function by a noncompetitive mechanism consistent with channel blockade.

Tubocurarine thus is unusual in that it competes with [3H]EB for the α3/β4 receptor’s agonist binding site with an apparent K_d of 23 μM (Fig. 4, Table 1), but at similar concentrations it blocks the receptor’s function by a noncompetitive mechanism, suggesting that it might be a channel blocker. Therefore, we investigated the mechanism of the inhibition by d-tubocurarine of [3H]EB binding to α3/β4 receptors in more detail by examining its effect on [3H]EB binding saturation curves and comparing it with the competitive antagonist DHβE. As shown in Fig. 7, the block of [3H]EB binding by 250 μM DHβE fit a model of competitive inhibition, shifting the apparent K_d value of [3H]EB by a factor of 4 with no change in its B_{max} (Fig. 7B). The block of [3H]EB binding by 30 μM d-tubocurarine, however, seemed to fit a model of a mixed mechanism. Competitive inhibition was indicated by

![Image](image_url)
the ~3-fold shift in apparent \(K_d\) for \([3H]EB\), but there also was a 25% decrease in the apparent \(B_{\text{max}}\) value for \([3H]EB\) binding in the presence of \(d\)-tubocurarine (Fig. 7B).

**Ca\(^{2+}\) imaging.** As shown in Fig. 8A, nicotine stimulated an increase in [Ca\(^{2+}\)], concentration in KXo3/b4R2 cells in a concentration-dependent manner. When the extracellular Ca\(^{2+}\) concentration was maintained at 1.3 mM, nicotine at concentrations of 10 and 100 \(\mu\)M increased [Ca\(^{2+}\)] levels to 2-fold and 5-fold of base-line, respectively. When the extracellular Ca\(^{2+}\) was raised from 1.3 to 10 mM (Fig. 8B), the basal level of [Ca\(^{2+}\)], increased slightly, but the increase in [Ca\(^{2+}\)], stimulated by 10 \(\mu\)M nicotine was markedly enhanced to >10-fold of base-line. This nicotine stimulated increase in [Ca\(^{2+}\)], was almost completely blocked by 10 \(\mu\)M mecamylamine (Fig. 8B).

**Na\(^{+}\) imaging.** Nicotine also stimulated an increase in [Na\(^{+}\)], measured by SBFI/Na\(^+\) imaging, and this increase was blocked by mecamylamine (Fig. 9A). Unlike changes in [Ca\(^{2+}\)], however, the nicotine-stimulated increase in [Na\(^{+}\)], was not affected by increasing the extracellular Ca\(^{2+}\) from 1.3 to 10 mM (Fig. 9B).

**Binding of \([3H]EB\) to cell surface receptors.** The KXo3/b4R2 cells produce a very high density of \(\alpha_3/\beta_4\) receptors, and the ligand binding studies indicate that these receptors have characteristics of a single homogenous population. However, ligand binding measurements in membrane homogenates do not distinguish between receptors on the cell surface membrane and receptors on intracellular membranes. Therefore, to estimate the fraction of the total \(\alpha_3/\beta_4\) receptor population that is located on the cell surface of KXo3/b4R2 cells, \([3H]EB\) binding was measured in intact cells attached to 24-well tissue culture plates, and nonspecific binding was determined with either nicotine (which easily crosses cell membranes) or carbachol (which does not readily cross cell membranes). As shown in Fig. 10, nicotine blocked >95% of the \([3H]EB\) binding sites in intact cells, whereas in contrast, the nonpermeant carbachol blocked a maximum of 40% of the \([3H]EB\) binding sites even at a concentration 25,000 times higher than its affinity constant (\(K_d\)) for the receptor, as determined in membrane binding assays (see Table 1). Thus, we estimate that 40% of the total number of \(\alpha_3/\beta_4\) receptors measured in KXo3/b4R2 cell homogenates are located on the cell surface.

![Fig. 8. Effects of nicotine on [Ca\(^{2+}\)] in KXo3/b4R2 cells. Changes in [Ca\(^{2+}\)], were measured and calculated as described in Experimental Procedures. Cells were loaded with the Ca\(^{2+}\) fluorescent indicator Fura-2 and then exposed to buffer alone or buffer containing nicotine with or without mecamylamine. Traces, [Ca\(^{2+}\)], changes. Concentrations of nicotinic drugs during the period indicated (horizontal bars) are marked (above traces). Extracellular Ca\(^{2+}\) concentration and time scales are shown (under each trace). Nic, \((-\)nicotine. Mec, mecamylamine. Ca, extracellular Ca\(^{2+}\) concentration. A, [Ca\(^{2+}\)], increase is stimulated by nicotine at 1.3 mM extracellular Ca\(^{2+}\). Data shown are representative of three independent experiments, and the trace shown is the average [Ca\(^{2+}\)], of 58 cells. B, [Ca\(^{2+}\)], increase is stimulated by nicotine and blocked by mecamylamine at an extracellular Ca\(^{2+}\) concentration of 10 mM. Data shown are representative of three independent experiments, and the trace shown is the average [Ca\(^{2+}\)], of 37 cells.](image)

![Fig. 9. Effects of nicotine on [Na\(^{+}\)], of KXo3/b4R2 cells. Relative changes in [Na\(^{+}\)], are shown in arbitrary units of fluorescence ratio F334/F380, as described in Experimental Procedures. Cells were loaded with the Na\(^{+}\) fluorescent indicator SBFI and then exposed to buffer containing nicotinic drugs. Traces, [Na\(^{+}\)], changes. Concentrations of nicotinic drugs during the period indicated (horizontal bars) are marked (above traces). Extracellular Ca\(^{2+}\) concentration and time scales were shown (under each trace). Nic, \((-\)nicotine. Mec, mecamylamine. Ca, extracellular Ca\(^{2+}\) concentration. A, [Na\(^{+}\)], increase is stimulated by nicotine and blocked by mecamylamine at 10 mM extracellular Ca\(^{2+}\). Data shown are representative of two independent experiments, and the trace shown is the average [Na\(^{+}\)], of 27 cells. B, [Na\(^{+}\)], increase is stimulated by nicotine at different extracellular Ca\(^{2+}\) concentrations. Data shown are representative of three independent experiments, and the trace shown is the average [Na\(^{+}\)], of 77 cells. Dotted horizontal lines, [Na\(^{+}\)], in cells that were permeabilized with 5 \(\mu\)M Gramicidin A when extracellular [Na\(^{+}\)] was 154 mM (top line) and 0 mM (bottom line), respectively.](image)
Discussion

The KXα3β4R2 cells described here stably express α3/β4 nAChRs that bind [3H]EB with high affinity and function to gate cations through their channels in response to nicotinic agonists. In contrast, cells expressing the mRNA for either the α3 or β4 subunit alone do not express [3H]EB binding sites or cation channels that respond to nicotine. Both [3H]EB saturation binding and drug competition studies indicate that KXα3β4R2 cells express a single class of nicotinic receptor binding sites; similarly, the studies of the receptor function are consistent with a single class of receptors. These cells produce a very high density of α3/β4 receptors, and binding studies in intact cells indicate that ~40% of the total number of receptors are located on the cell surface. Only the receptors on the cell surface would be expected to mediate functional responses, whereas receptors on intracellular membranes might be in various stages of their receptor cycle, either before insertion into the cell surface membrane or after removal from it. Furthermore, we cannot exclude the possibility that these cells express receptors with more than one stoichiometric combination of α3 and β4 subunits but very similar pharmacological and functional properties. Nevertheless, these cells provide a model system in which the pharmacology, function, and regulation of the α3/β4 nAChR can be examined in detail.

Drug binding competition studies indicated that the affinity of every agonist examined is lower at the α3/β4 receptor binding site in these cells than at the α4/β2 nicotinic receptor site in rat forebrain, with affinity ratios ranging from 7 for EB to >750 for A85380. One consequence of the lower affinity of agonists for α3/β4 receptors is that with the exception of [3H]EB, radioligands such as [3H]cytisine, [3H]nicotine, and [3H]acetylcholine, which have been very useful for labeling the α4/β2 nAChR, the predominant receptor subtype in brain, probably would not be useful for labeling α3/β4 receptors. This is because their lower affinities would require ligand concentrations (200–880 nM) at which nonspecific binding would obscure specific binding. This probably explains why these radioligands have not been useful for labeling nicotinic receptors in autonomic ganglia or adrenal gland, where a receptor subtype containing α3 subunits seems to predominate. Although the affinity of EB also is lower at α3/β4 receptors than at α4/β2 receptors in brain, its affinity at these α3/β4 receptors (300 pM) is still very high, making it an excellent radioligand for these receptors.

The affinities of d-tubocurarine and DHβE, the two antagonists examined that competed for the binding site, like those of the agonists, are lower at the α3/β4 receptor binding site than at the α4/β2 binding site in brain. In fact, DHβE displays the largest difference in affinity for these two receptor subtypes of any drug examined. Thus, DHβE, with a receptor binding site affinity ratio of >7500, and A85380, with an affinity ratio of >750, could be useful drugs for distinguishing between these two subtypes of nicotinic receptors in native tissues. In addition to the absolute difference in affinity of DHβE, a second way to distinguish between these two receptor subtypes could be to take advantage of the difference in the rank orders of affinities of d-tubocurarine and DHβE. Thus, at α3/β4 receptor binding sites, d-tubocurarine has >9 times higher affinity than DHβE, whereas in contrast, at α4/β2 binding sites, DHβE has >75 times higher affinity than d-tubocurarine.

Consistent with a previous report (Houghtling et al., 1995), both d-tubocurarine and DHβE show low Hill coefficients in competing for brain [3H]EB binding sites, which are predominantly α4β2 receptors. Similarly, d-tubocurarine shows a low Hill coefficient in competing for α3/β4 receptors in these transfected cells. These low Hill coefficients suggest the interesting possibility that some antagonists can distinguish between two states of the same receptor, for example, as the receptor conformation changed from a resting state to a desensitized state (with high affinity for agonists) during prolonged incubation with an agonist, as in the receptor binding assay.

The α3/β4 nAChR function and its pharmacology were assessed by measuring agonist-stimulated 86Rb+ efflux. Nic-
otine and acetylcholine both stimulated $^{86}\text{Rb}^+$ efflux in a concentration-dependent manner, with EC$_{50}$ values of $\sim 28$ and $114 \mu M$, respectively. The difference between the affinity of the receptor for these agonists as assessed by this measurement of receptor function compared with binding studies (Table 1) presumably reflects the shift of the receptor to a state with high affinity for agonists under equilibrium binding conditions. In the cases of nicotine and acetylcholine, the EC$_{50}$ values for functional activation were 59- and 129-fold higher, respectively, than the $K_v$ values measured in binding studies.

Among the antagonists tested, mecamylamine, with an IC$_{50}$ value of 1 $\mu M$, was the most potent in blocking nicotine-stimulated $^{86}\text{Rb}^+$ efflux in KXa3/4R2 cells. It was $\sim 10$ times more potent than $d$-tubocurarine, 100 times more potent than DHAB, and 200 times more potent than hexamethonium. Both mecamylamine and hexamethonium blocked receptor function by a noncompetitive mechanism, suggesting that they block the receptor ion channel. This would explain their effective block of receptor function in the face of their near-total inability to compete for the agonist binding site of the $\alpha$3/4 receptor.

Tubocurarine, on the other hand, presents a more interesting case. In the binding assay, it competes for the $\alpha$3/4 receptor agonist recognition site with a $K_v$ value of $\sim 23 \mu M$ (Table 1), but at similar concentrations, its blockade of receptor function seems to be entirely by a noncompetitive mechanism. In fact, there was no indication that $d$-tubocurarine competes for the agonist binding site in the $^{86}\text{Rb}^+$ efflux assay; that is, the concentration-response curves for nicotine were not shifted to the right in the presence of any concentration of tubocurarine tested (Fig. 6C). One explanation for this apparent contradiction between the binding assay and the $^{86}\text{Rb}^+$ efflux assay is that in the receptor’s functional state, as measured in the $^{86}\text{Rb}^+$ efflux assay, tubocurarine has virtually no affinity for the agonist recognition site of the receptor but nevertheless very effectively blocks receptor function noncompetitively by blocking the ion channel. However, when the receptor conformation shifts to a desensitized state with its higher affinity for nicotinic agonists, as occurs in the binding assay, its affinity for tubocurarine also increases, enabling this antagonist to then compete with micromolar affinity for the agonist recognition site. This explanation also can account for the observation that tubocurarine competes for the agonist recognition sites of both the $\alpha$3/4 receptor in these cells and the $\alpha$4/2 receptor in rat brain with a low Hill coefficient because this could reflect the different affinities of the drug for the different conformations of the receptors. In fact, the $[H]E$ saturation binding studies shown in Fig. 7 suggest that in the presence of 30 $\mu M$ tubocurarine, $\sim 25\%$ of the receptors may remain in a conformation with an affinity for $[H]E$ too low to be measured in a typical binding assay.

In contrast to these other antagonists, DHAB blocked receptor function in a competitive manner. Its affinity constant for the functional state of the receptor, calculated from its IC$_{50}$ value in blocking nicotine-stimulated $^{86}\text{Rb}^+$ efflux (after correction for the nicotine concentration), is $\sim 20 \mu M$, which is $\sim 10$ times lower than the $K_v$ value derived from the ligand binding competition assays (Table 1). In other words, DHAB is $\sim 10$ times more potent in blocking receptor function than would have been predicted from the binding assay. This suggests that DHAB has higher affinity for the functional conformation of the receptor than for its conformation in the desensitized state, which would occur during the binding assay.

Activation of the $\alpha$3/4 receptor in KXa3/4R2 cells resulted in an increase in both $[Ca^{2+}]_i$ and $[Na^+]_i$. The nicotine-stimulated increase in $[Ca^{2+}]_i$ was markedly enhanced when the concentration gradient was increased by raising the extracellular $Ca^{2+}$ concentration from 1.3 to 10 mM. The higher extracellular $Ca^{2+}$ concentration, however, seemed to have no effect on the nicotine-stimulated increase in $[Na^+]_i$. These results suggest that extracellular $Ca^{2+}$ can actually pass through the $\alpha$3/4 receptor channel.

The $\alpha$3/4 nAChR has been expressed previously in HEK 293 cells, both transiently (Wong et al., 1995) and stably in conjunction with a voltage-gated $Ca^{2+}$ channel (Stetzer et al., 1996). Although pharmacological comparisons of these previously expressed $\alpha$3/4 nAChRs and those in the KXa3/4R2 cells are very limited, there seems to be agreement that acetylcholine, nicotine, and 4-cyanopiperidine are full agonists, confirming studies in frog oocytes (Luettje and Patrick, 1991; Covernton et al., 1994; Papke and Heinemann, 1994). However, nicotine and acetylcholine seemed to be somewhat more potent in stimulating the $\alpha$3/4 receptors in these KXa3/4R2 cells than was reported in the other transfected HEK 293 cells.

In conclusion, KXa3/4R2 cells provide a model system in which to study $\alpha$3/4 receptors, a subtype that may mediate neurotransmission in autonomic ganglia and brain. These cells have maintained a high level of receptor expression and function for $\geq$300 generations and thus have allowed a detailed examination of the pharmacology of the receptor. The pharmacological studies reported here, for both drug binding and functional activation, should help in studying the distribution, physiological role, and pharmacology of $\alpha$3/4 nAChRs in brain and peripheral tissues. In addition, this cell line provides a stable and convenient source of highly functional channels for patch-clamp experiments to study in detail the electrophysiological properties of $\alpha$3/4 nAChRs (Zhang et al., 1997). Furthermore, the binding site of the receptor and its function in these cells are altered by exposure to nicotinic drugs (Meyer et al., 1997); thus, these stably transfected cells should prove useful in studying the chronic and acute effects of nicotinic drugs on the density, cellular distribution, and function of $\alpha$3/4 nAChRs.

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