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# Agonist Regulation of Rat $\alpha3\beta4$ Nicotinic Acetylcholine Receptors Stably Expressed in Human Embryonic Kidney 293 Cells

ERIN L. MEYER, YINGXIAN XIAO, and KENNETH J. KELLAR

*Department of Pharmacology, Georgetown University School of Medicine, Washington, DC*

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## ABSTRACT

Effects of agonists on rat $\alpha3\beta4$ nicotinic acetylcholine receptors expressed in KX$\alpha3\beta4$R2 cells [human embryonic kidney 293-derived cells] were studied. The potencies of seven agonists varied over a 7000-fold range, with a rank order of epibatidine $\gg$ A85380 $>$ cytisine $\approx$ 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) $\approx$ nicotine $>$ acetylcholine $>$ carbachol. The efficacies of all of the agonists studied here were similar except for DMPP, which seemed to be a partial agonist compared with nicotine and acetylcholine. Nicotine and carbachol desensitized the receptors in a time- and concentration-dependent manner. The $EC_{50}$ values for nicotine and carbachol to desensitize the receptors during a 60-min exposure were 3 and 51 $\mu$M, respectively, indicating that these agonists are more potent at desensitizing the receptors than at activating them. The function of the receptors recovered from agonist-induced desensitization rapidly and almost completely. The half-time for recovery of function from desensitization after a 60-min treatment with nicotine increased with the concentration of nicotine used to desensitize the receptors. In contrast, no such concentration dependence for time to recovery of function was found when carbachol was used to desensitize the receptors. We propose that this difference may be due to the cell permeability of nicotine, allowing it to enter and be sequestered inside of cells and then slowly diffuse out to maintain receptor desensitization. After a 5-day exposure to 100 $\mu$M nicotine, the receptors were completely desensitized, but receptor function recovered to 83% of control values with a half-time of about 10.5 min. Although the number of nicotinic receptor binding sites measured with (±)-[$^3$H]epibatidine was increased during the chronic treatment with nicotine, no increase in function was detected.





Neuronal nicotinic receptors are expressed throughout the CNS and peripheral nervous system. These receptors are composed of $\alpha$ and $\beta$ subunits and exist as subtypes defined by their particular subunit composition. Nine different $\alpha$ subunits ($\alpha2$–$\alpha10$) and three different $\beta$ subunits ($\beta2$–$\beta4$) have been identified in vertebrates to date, indicating significant structural diversity among these receptor subtypes. The range of functional properties and pharmacological characteristics among these receptors is primarily a direct reflection of this structural diversity.

One putative nicotinic receptor is the $\alpha3\beta4$ subtype, composed of $\alpha3$ and $\beta4$ subunits. This subtype or a close variant of it may be one of the major nicotinic receptors in some autonomic ganglia (Conroy and Berg, 1995; Wong et al., 1995; Poth et al., 1997), sensory ganglia (Flores et al., 1996), and adrenal gland (Campos-Caro et al., 1997), as well as in several important regions of the CNS (Mulle et al., 1991; Winzer-Serhan and Leslie, 1997; Zoli et al., 1998; Quick et al., 1999).

Recently, we established a transfected HEK 293 clonal cell line, KX$\alpha3\beta4$R2, that stably expresses a high density of functional $\alpha3\beta4$ receptors (Xiao et al., 1998; Zhang et al., 1999). We have used these cells to characterize the pharmacology of the agonist binding site and the function of the $\alpha3\beta4$ nicotinic receptor (Xiao et al., 1998). We found, for example, that although all the nicotinic agonists examined have relatively high affinity for the $\alpha3\beta4$ receptor binding sites, their affinities were much lower than in rat forebrain, in which $\alpha4\beta2$ receptors predominate (Whiting and Lindstrom, 1987; Flores et al., 1992). In fact, the equilibrium dissociation constants for acetylcholine, nicotine, and cytisine at $\alpha3\beta4$ receptors are 200 to 800 nM (Xiao et al., 1998). These affinities are far too low to allow radioactive versions of these agonists to effec-

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**ABBREVIATIONS:** CNS, central nervous system; $^{86}Rb^+$, [$^{86}$Rb]rubidium chloride; EB, (±)-epibatidine; HEK, human embryonic kidney; nAChR, nicotinic acetylcholine receptor; DMPP, 1,1-dimethyl-4-phenyl-piperazinium iodide.

tively label these receptors directly, which probably explains why these ligands have, in general, not been very useful as radioligands for measuring the nicotinic receptors in ganglia and adrenal gland, in which α3β4 receptors may predominate. In contrast, epibatidine binds to these heterologously expressed α3β4 receptors with a dissociation constant of about 300 pM and is an excellent radioligand for measuring them, as well as the receptors in adrenal gland, autonomic, and central ganglia (Houghtling et al., 1995; Flores et al., 1996; Dávila-García et al., 1997).

In addition to information about the receptor binding site, studies in these transfected cells showed that receptor function could be assessed by measurements of [$^{86}$Rb]rubidium chloride ($^{86}Rb^+$) ion efflux, $Ca^{2+}$ ion imaging, $Na^+$ ion imaging, and with whole-cell patch-clamp methods (Xiao et al., 1998; Zhang et al., 1999; Hernandez et al., 2000). These studies indicated that acetylcholine and nicotine are equally efficacious at activating α3β4 receptors; that mecamylamine, hexamethonium, and *d*-tubocurarine are effective noncompetitive blockers; and that dihydro-β-erythroidine is a competitive blocker of α3β4 receptors (Xiao et al., 1998).

Among the important properties of nicotinic receptors are their propensity to desensitize during exposure to agonists and their rate of recovery from desensitization. These properties vary among the different receptor subtypes and may be critical in determining which nicotinic receptors are able to respond to stimulation by endogenous acetylcholine or exogenous agonists and to what degree they can respond. Both α and β subunits seem to contribute to the characteristics of desensitization of nicotinic receptors expressed heterologously in oocytes (Cachelin and Jaggi, 1991; Gross et al., 1991). We have used the KXα3β4R2 cells to further study agonist effects at α3β4 receptors and to characterize their desensitization and recovery from desensitization after short- and long-term exposure to nicotinic agonists.

## Experimental Procedures

**Materials and Drugs.** Tissue culture medium, fetal bovine serum and antibiotics were obtained from Invitrogen (Carlsbad, CA). (±)-[$^3$H]epibatidine ([$^3$H]EB) and $^{86}Rb^+$ were supplied by PerkinElmer Life Science Products (Boston, MA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated.

**Cell Culture.** The cell line KXα3β4R2 was established previously by stably cotransfecting HEK 293 cells with the rat α3 and β4 nAChR subunit genes (Xiao et al., 1998). KXα3β4R2 cells were grown as described previously (Xiao et al., 1998) in minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.7 mg/ml Geneticin (G418) at 37°C with 5% $CO_2$ in a humidified incubator.

**$^{86}Rb^+$ Efflux Assay.** Functional properties of the nAChRs expressed in the KXα3β4R2 cells were assessed by measurements of nicotinic agonist-stimulated $^{86}Rb^+$ efflux, as described previously (Xiao et al., 1998). In brief, aliquots of cells in the selection growth medium were plated into 24-well plates coated with poly-D-lysine. The plated cells were grown at 37°C for 18 to 24 h to reach 70 to 95% confluence. The cells were then incubated in growth medium (0.5 ml/well) containing $^{86}$RbCl (2 μCi/ml) for 4 h at 37°C, the loading mixture was aspirated, and the cells were washed four times with HEPES 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; 1 ml/well). One milliliter of buffer, with or without agonists, was then added to each well. After incubation for 2 min, the assay buffer was collected and the amount of $^{86}Rb^+$ in the buffer was determined. Cells were then lysed by adding 1 ml of 100 mM NaOH to each well, and the lysate was collected for determination of the amount of $^{86}Rb^+$ in the cells at the end of the efflux assay. Radioactivity of assay buffer samples and lysates was measured by liquid scintillation counting. Total amount of $^{86}Rb^+$ loaded (counts per minute) was calculated as the sum of the assay buffer sample and the lysate of each 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 of nicotinic agonists and basal efflux measured in the absence of agonists. Basal $^{86}Rb^+$ efflux ranged from 3 to 6% and maximal stimulated efflux was approximately 45% of loaded $^{86}Rb^+$. Nonlinear regression analyses and statistical analyses were performed using Prism software (GraphPad Software, San Diego, CA).

In assays to measure agonist-induced desensitization and recovery of receptor function, agonists were added to the $^{86}Rb^+$ loading media and the wash buffer for the times indicated in the figure legends. Nicotine-stimulated $^{86}Rb^+$ efflux was then either measured immediately after removal of the desensitizing agonists or after the periods of recovery indicated in the figure legends. In most assays to measure desensitization and recovery, the amount of $^{86}Rb^+$ loaded in presence of desensitizing agonists was not significantly diminished by agonist-induced efflux because the ion, which is taken up by a sodium-potassium ATPase, continues to enter the cell long after receptor desensitization has occurred. However, after short periods of agonist-induced desensitization (e.g., less than 10 min in the experiment shown in Fig. 2), the amount of $^{86}Rb^+$ remaining in the cells at the start of the measurements of receptor function was reduced by about 40% compared with control cells loaded in the absence of agonists. To account for this, as well as the variations in the amount of cells in each well, basal and receptor-mediated efflux were normalized to the amount of $^{86}Rb^+$ loaded, as described above. We have found that, over a wide range of loaded $^{86}Rb^+$ (20,000–200,000 cpm/well), the amount of basal and nicotine-stimulated $^{86}Rb^+$ efflux is proportional to the amount of the ion in the cell at the beginning of stimulation.

The $^{86}Rb^+$ efflux assay obviously does not have the temporal resolution of patch clamp measurements; over the 2-min period during which the measurements were made, however, nicotine-stimulated efflux from these cells reflects receptor stimulation with a high degree of reproducibility. Moreover, we find essentially the same concentration-response relationships for nicotinic agonist stimulation of $^{86}Rb^+$ efflux from these cells whether we use a 1- or 2-min stimulation period (Y. Xiao and K. J. Kellar, unpublished observations).

**Radioligand Binding Assay.** To measure effects of long-term treatment with nicotine on α3β4 receptor density, cells were cultured in the presence or absence of nicotine for the times indicated. Binding of [$^3$H]EB to receptors was then measured as described previously (Xiao et al., 1998), with minor modifications. Briefly, the cells were harvested in 50 mM Tris·HCl, pH 7.4, washed, homogenized with the use of Brinkmann polytron homogenizer (Brinkmann Instruments, Westbury, NY), and centrifuged at 35,000*g*. The pellets were washed three times by suspension in fresh buffer and centrifugation at 35,000*g*. The resulting washed membranes were then incubated with approximately 3 nM [$^3$H]EB for 4 h at 24°C in a final volume of 1 ml. Nonspecific binding was assessed in parallel incubations in the presence of 300 μM nicotine. Bound and free ligands were separated by vacuum filtration through Whatman GF/C filters (Whatman, Clifton, NJ) treated with 0.5% polyethylenimine. The filter-retained radioactivity was measured by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding.

## Results

**Agonist Stimulation of α3β4 Nicotinic Receptors.** Concentration response curves for seven nicotinic agonists

stimulating $^{86}Rb^+$ efflux via $\alpha3\beta4$ receptors in KX$\alpha3\beta4$R2 cells are shown in Fig. 1 and the potency ($EC_{50}$) and relative efficacy ($E_{max}$) for each drug is shown in Table 1. The potencies of the agonists varied over a 7,000-fold range, from 60 nM for EB to 440 $\mu$M for carbachol (Table 1). The rank order of potency for stimulating $^{86}Rb^+$ efflux in these studies was EB $\gg$ A85380 $>$ cytisine $\approx$ DMPP $\approx$ nicotine $>$ acetylcholine $>$ carbachol (Table 1). Nicotine was approximately 4 times more potent than acetylcholine but 500 times less potent than EB. Interestingly, A85380, which has very high affinity for $\alpha4\beta2$ nicotinic receptor binding sites (Sullivan et al., 1996; Xiao et al., 1998; Mukhin et al., 2000) but about 750 times lower affinity for $\alpha3\beta4$ receptor binding sites (Xiao et al., 1998; Mukhin et al., 2000), was nevertheless the second most potent agonist at activating $\alpha3\beta4$ receptors; in fact, A85380 was about 5 times more potent than nicotine, although it was still about 90 times less potent than EB.

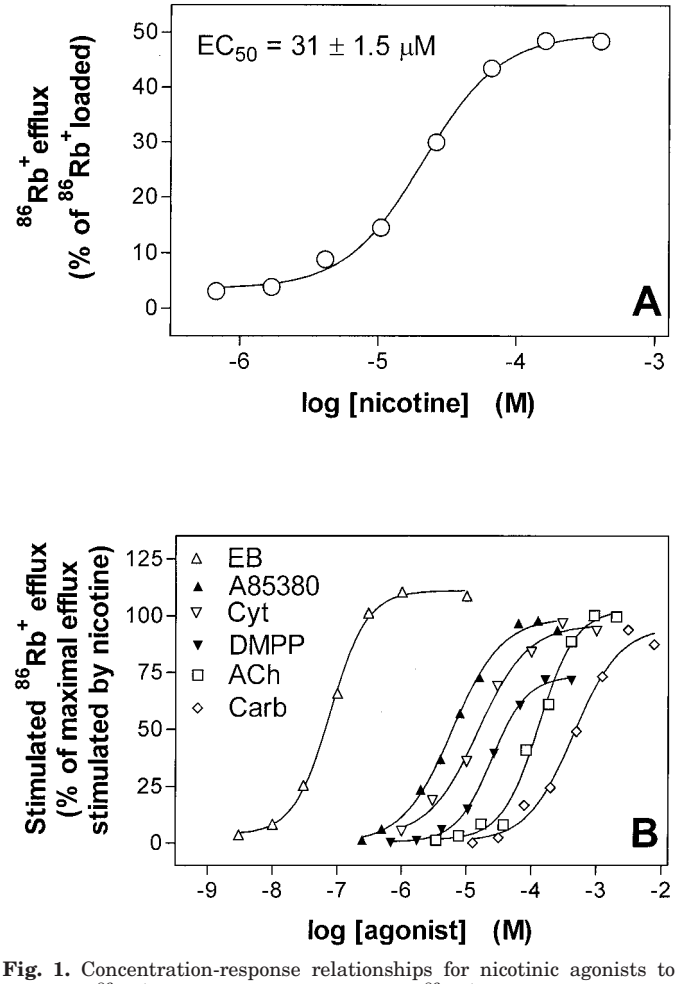


**Fig. 1.** Concentration-response relationships for nicotinic agonists to stimulate $^{86}Rb^+$ efflux from KX$\alpha3\beta4$R2 cells. $^{86}Rb^+$ efflux was measured as described under *Experimental Procedures*. The data were fit to the equation for a sigmoidal concentration-response relationship. Each data point represents the mean of quadruplicate determinations. A, concentration-response curve for nicotine-stimulated $^{86}Rb^+$ efflux. The amount of $^{86}Rb^+$ efflux was expressed as percentage of total $^{86}Rb^+$ loaded. Data from a representative experiment are shown. B, comparison of concentration-response relationships of different nicotinic agonists. To account for variations among experiments done on different days, data were expressed as a percentage of the maximum response to nicotine, which was determined with each group of assays. Baseline efflux (≈5%) has been subtracted. Data from a single set of representative experiments are shown. See Table 1 for $EC_{50}$ and $E_{max}$ values from all experiments. Carb, carbachol; Cyt, cytisine.

Most of the agonists tested here, including cytisine, stimulated a maximum response similar to that of nicotine and acetylcholine. The exception was DMPP, which acted as a partial agonist producing a maximum response approximately 67% of that elicited by nicotine ($P < 0.05$). The apparently slightly greater maximum response to epibatidine compared with nicotine may result from its very high potency, which allows it to evoke a maximal response before any significant agonist blockade of the channel occurs (Xiao et al., 2000a).

**Time Course- and Concentration-Dependence of $\alpha3\beta4$ Receptor Desensitization by Agonists.** Nicotinic receptors desensitize during exposure to agonists. This loss of responsiveness is usually both time- and concentration-dependent (Marks et al., 1994), but these parameters vary according to the receptor subtype (Fenster et al., 1997). To examine the time course of desensitization of the $\alpha3\beta4$ receptors, we treated the cells with nicotine for different time periods and then, after removing the nicotine-containing media, immediately measured the receptor-mediated $^{86}Rb^+$ efflux response elicited by 100 $\mu$M nicotine, a nearly maximally effective concentration. When the receptor response was measured immediately after exposure to 10 or 100 $\mu$M nicotine for different time periods, the response decreased in an exponential manner with half-times of about 11 min after exposure to 10 $\mu$M nicotine and 1 min after exposure to 100 $\mu$M nicotine (Fig. 2). The response to nicotine was decreased by approximately 90% after exposure to 10 $\mu$M nicotine for 60 min, and by at least 94% after exposure to 100 $\mu$M nicotine for 60 min (Fig. 2). In contrast, after exposure to 1 $\mu$M nicotine for 60 min, the response was decreased by only about 27% (Fig. 2), and nonlinear regression analysis projected it to reach plateau at approximately 58% of control with a half-time of 41 min.

The concentration-dependence of desensitization of $\alpha3\beta4$ receptors by nicotine and by carbachol was examined by treating cells with a wide range of concentrations of these agonists for 60 min and then, immediately after removing the agonists, measuring the $^{86}Rb^+$ efflux response elicited by 100 $\mu$M nicotine. As shown in Fig. 3, the $EC_{50}$ values for nicotine and carbachol to desensitize these $\alpha3\beta4$ receptors during the 60-min exposure were about 3 and 51 $\mu$M, respectively, and receptor function was completely eliminated by treatment with 100 $\mu$M nicotine or 1000 $\mu$M carbachol. For both drugs, the slope of the Hill function was close to 1 (Fig. 3).

**Recovery from Desensitization.** We next examined the rate at which these $\alpha3\beta4$ nicotinic receptors recover from

TABLE 1

Comparison of pharmacological properties of nAChR agonist stimulation of $^{86}Rb^+$ efflux from KX$\alpha3\beta4$R2 cells

Values shown are the mean ± S.E.M. of at least three independent experiments measured in quadruplicate. See Figure 1 for description of data analyses and curve fittings.

| Agonist | $EC_{50}$ | Relative $E_{max}$ |
|---|---|---|
| | *μM* | *% of nicotine $E_{max}$* |
| (±)EB | 0.06 ± 0.01 | 112 ± 1 |
| A-85380 | 5.7 ± 0.3 | 101 ± 2 |
| Cytisine | 24 ± 7.4 | 90 ± 4 |
| DMPP | 28 ± 3.9 | 67 ± 7* |
| (−)-Nicotine | 31 ± 1.5 | 100 ± 9 |
| Acetylcholine | 110 ± 11 | 99 ± 3 |
| Carbachol | 442 ± 81 | 86 ± 5 |

* Different from nicotine ($P < 0.05$ by unpaired $t$ test).

desensitization and whether that rate is dependent upon the concentration of the agonist that induced the desensitization. The receptors were desensitized by incubation of the cells with either 100 or 300 μM nicotine or 1000 or 3000 μM carbachol for 60 min. The agonists were then removed and $^{86}Rb^+$ efflux stimulated by 100 μM nicotine was measured either immediately or after the cells were washed and allowed to recover in buffer for increasing amounts of time.

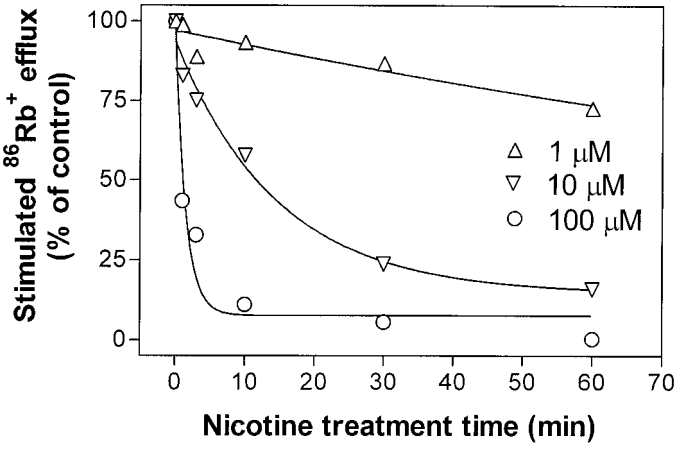


**Fig. 2.** Time course of nicotine-induced desensitization of α3β4 receptor function. Cells were treated with 1, 10, or 100 μM nicotine for the times shown during the end of the $^{86}Rb^+$ loading procedure and/or during the four washes to remove the $^{86}Rb^+$ not taken up by the cells. Immediately after removing the last nicotine-containing wash buffer, fresh buffer containing 100 μM nicotine was added for 2 min to measure nicotine-stimulated $^{86}Rb^+$ efflux, as described under *Experimental Procedures*. Results are expressed as the percentage of control samples incubated for the same times in media alone before measuring nicotine-stimulated efflux. The nicotine-stimulated response decreased with time of prior treatment with nicotine as a single exponential function described by $Y = Y_{max}e^{-kt}$. Half-time of the decay was calculated by $t_{1/2} = 0.693/k$. Each data point represents the mean of quadruplicate determinations. The results shown are from a single representative experiment. The half-times for desensitization after exposure to 10 and 100 μM nicotine were 11.1 ± 0.4 min and 1.3 ± 0.1 min, respectively (mean ± S.E.M., $n = 4$). After exposure to 1 μM nicotine, function was projected to reach plateau at 58% of control with a half-time ≈41 min ($n = 1$).

Immediately after the 60-min exposure to 100 or 300 μM nicotine, the response to the subsequent test stimulation with 100 μM nicotine was reduced by at least 95% (see Fig. 3). As shown in Fig. 4, after the nicotine was removed and the cells were washed four times, the sensitivity of the α3β4 nicotinic receptors began to return almost immediately, and within 40 min in recovery buffer reached about 75% of the response measured in control cells that had been preincubated for 60 min in the absence of nicotine. The recovery of receptor response after the 60-min exposure to 100 and 300 μM nicotine seemed to reach a plateau at approximately 93% and 83% of control responses, respectively (Table 2 and Fig. 4). However, the rate of return of receptor sensitivity was related to the concentration of nicotine used to induce desensitization (Fig. 4). Thus, as shown in Table 2, the half-times for the recovery of the nicotinic receptor-mediated response after cells were treated with 100 or 300 μM nicotine were 7 or 12 min, respectively ($P < 0.01$).

Because nicotine easily crosses cell membranes and thus may be sequestered within the cells, it is possible that the slower return of function after exposure to the higher concentrations of nicotine reflects a slower effective removal of the drug. If this were the case, as nicotine left the cells during washout, it might reach extracellular concentrations high enough to rebind a significant number of surface receptors and prolong desensitization. To address this possibility, we examined desensitization and recovery of receptor function after exposure of cells for 60 min to carbachol, which, as a

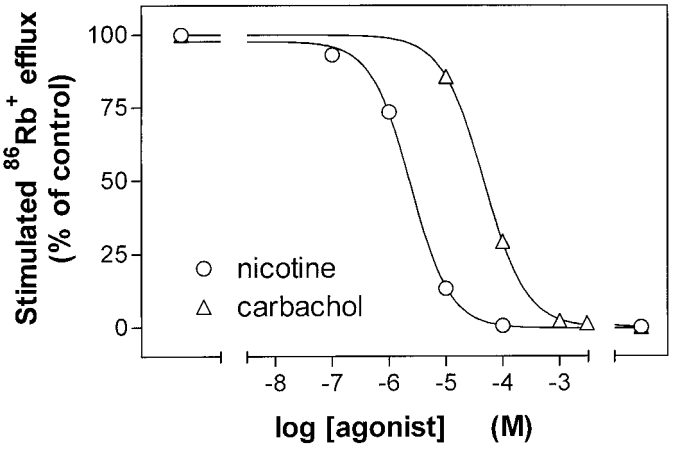


**Fig. 3.** Concentration-dependence for desensitization of α3β4 receptors by treatment with nicotine and carbachol for 60 min. Cells were treated with nicotine or carbachol at the concentrations shown for 60 min (during the last 50 min of the $^{86}Rb^+$ loading procedure and during a 10-min washing procedure). Immediately after removing the last wash buffer, fresh buffer containing 100 μM nicotine was added for 2 min to measure nicotine-stimulated $^{86}Rb^+$ efflux, as described under *Experimental Procedures*. Results are expressed as a percentage of nicotine-stimulated efflux in control samples, which were loaded with $^{86}Rb^+$ and washed in the absence of nicotine. Data were fit to the equation for a sigmoidal concentration-response relationship. The results shown are from a single representative experiment carried out in quadruplicate. The $EC_{50}$ values (mean ± S.E.M.) for induction of desensitization by nicotine and carbachol were 3.2 ± 0.3 μM ($n = 7$) and 51 ± 6 μM ($n = 5$), respectively. The Hill slopes were 1.1 ± 0.1 and 1.2 ± 0.1, respectively.

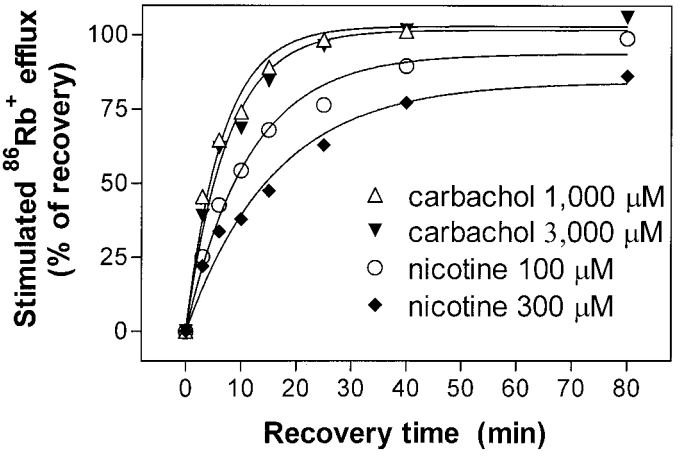


**Fig. 4.** Time course of recovery of α3β4 receptor function after desensitization by 60-min treatment with nicotine or carbachol. Cells were treated with nicotine (100 or 300 μM) or carbachol (1000 or 3000 μM) for a total time of 60 min. They were then washed rapidly three times in fresh buffer and incubated in a fourth wash buffer to allow recovery. At the times shown, the last wash buffer was removed, and nicotine-stimulated $^{86}Rb^+$ efflux was measured. To measure the responses at the 0 time point (no recovery), agonists were included in the wash buffer and nicotine-stimulated $^{86}Rb^+$ efflux was measured immediately after the fourth wash. Results are expressed as a percentage of recovery of function, which was based on the nicotine-stimulated efflux in control samples incubated for the same time periods in buffer alone. The recovery of receptor function increased with time as a single exponential function described by $Y = Y_{max}$ (1-$e^{-kt}$). The results shown are from a single representative experiment measured in quadruplicate. The experiment was repeated five times. See Table 2 for the half-times of recovery.

charged quaternary ammonium compound, does not readily cross cell membranes. Immediately after the 60-min exposure to 1000 or 3000 $\mu$M carbachol, function was reduced by at least 95% (see Fig. 3). As with nicotine, after carbachol was removed and the cells were washed, receptor function began to return almost immediately (Fig. 4). But compared with nicotine, recovery of function was faster and more complete, returning to essentially 100% of control responses within 80 min (Fig. 4 and Table 2). Moreover, in contrast to the rate of recovery of function after exposure to nicotine, the rate of recovery after carbachol was essentially independent of the carbachol concentration used to induce desensitization (Fig. 4). Thus, the half-times for recovery of nicotinic receptor function after exposure to 1000 or 3000 $\mu$M carbachol were estimated to be 3.5 or 4.1 min, respectively (Table 2). These recovery rates are not different from each other, but both are significantly faster than the rate after 300 $\mu$M nicotine ($P < 0.01$).

**Effects of Chronic Treatments with Nicotine on $\alpha3\beta4$ Receptor Function.** Prolonged exposure of some nicotinic receptors to high concentrations of nicotinic agonists is sometimes associated with loss of function that persists well beyond the presumed removal of the agonist. This phenomenon, referred to as receptor inactivation (Aoshima, 1984), has been seen in a wide variety of cell types. To determine whether these $\alpha3\beta4$ receptors are subject to inactivation, we grew cells in medium containing nicotine for up to 5 days before measuring receptor function. As expected based on the results from the 60-min exposure to nicotine shown in Fig. 3, when cells were exposed to 100 $\mu$M nicotine for 1, 3, or 5 days and assayed immediately after removal of the nicotine-containing medium, nicotine-stimulated $^{86}Rb^+$ efflux was essentially abolished (Fig. 5). Studies of the concentration-dependence of this loss of receptor function during a 5-day exposure to nicotine yielded an $EC_{50}$ value of 1.3 $\pm$ 0.1 $\mu$M (Fig. 6), which, although statistically different ($p < 0.01$) from that seen after incubation with nicotine for 60 min (3.2 $\pm$ 0.3 $\mu$M; see legend to Fig. 3), is surprisingly similar considering the different times of exposure. An important test for inactivation of receptors is whether or not the loss of receptor function is irreversible over a period of time that would exclude synthesis of new receptors as an explanation for return of function. To examine this, we grew cells for 5 days in the presence of 100 $\mu$M nicotine and then measured nicotine-stimulated function after washing the cells and allowing them to recover in nicotine-free medium for increasing periods of time, up to 1 day. Immediately after this 5-day exposure to nicotine, no receptor function was measurable. However, as shown in Fig. 7, even after this prolonged exposure to nicotine, receptor function began to return within a few minutes in recovery buffer and reached approximately 80% of the responses in control cells within 2 h. The half-time for return of function was 11 $\pm$ 0.6 min, which, although statistically different ($P < 0.01$) from that seen after treatment of cells with the same concentration of nicotine for only 60 min (7.0 $\pm$ 0.5 min), is surprisingly similar. Recovery of receptor function after 5 days' exposure to nicotine was incomplete (Fig. 7), and even after 24 h of recovery in nicotine-free medium,

TABLE 2

Comparison of recovery of $\alpha3\beta4$ receptor function after 60-min treatments with nicotine or carbachol

Values shown are the mean $\pm$ S.E.M. of five independent experiments measured in quadruplicate. See Figure 4 for description of experimental procedure and curve fittings.

| Agonist | Agonist concentration for 60-min treatment | Half-time for recovery | Relative maximum recovery |
|---|---|---|---|
| | $\mu M$ | *min* | *% of non-treated cells* |
| (−)-Nicotine | 100 | 7.0 $\pm$ 0.5 | 93 $\pm$ 2 |
| (−)-Nicotine | 300 | 12 $\pm$ 2* | 83 $\pm$ 4 |
| Carbachol | 1000 | 3.5 $\pm$ 0.5 | 103 $\pm$ 3 |
| Carbachol | 3000 | 4.1 $\pm$ 0.5 | 98 $\pm$ 2 |

* $P < 0.01$ compared with half-time for recovery after 100 $\mu$M nicotine or after 1000 or 3000 $\mu$M carbachol.

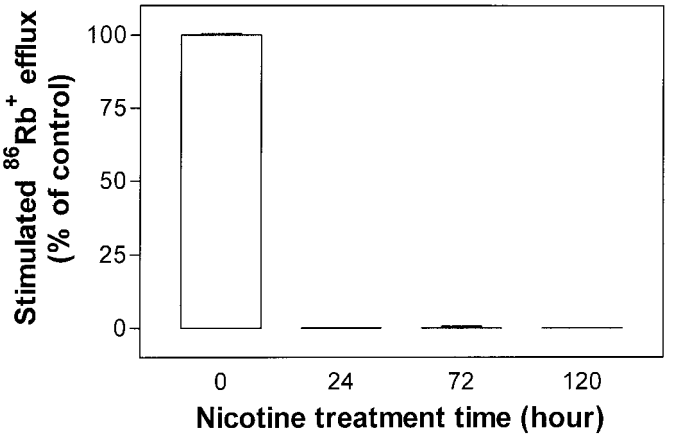


**Fig. 5.** Loss of $\alpha3\beta4$ receptor function after long-term treatment with 100 $\mu$M nicotine. Cells were grown in culture medium containing 100 $\mu$M nicotine for 24, 72, or 120 h. They were then loaded with $^{86}$RbCl for 4 h (still in the presence of nicotine). After removing the nicotine-containing loading medium, the cells were washed three times with buffer containing nicotine. Fresh buffer containing 100 $\mu$M nicotine was then immediately added for 2 min to measure nicotine-stimulated $^{86}Rb^+$ efflux. Results are expressed as a percentage of control cells never exposed to nicotine before measurement of nicotine-stimulated efflux. No nicotine-stimulated efflux was measurable in cells grown for 24, 72, or 120 h in the presence of 100 $\mu$M nicotine. Values are mean $\pm$ S.E.M. from three independent measurements that were made in quadruplicate.

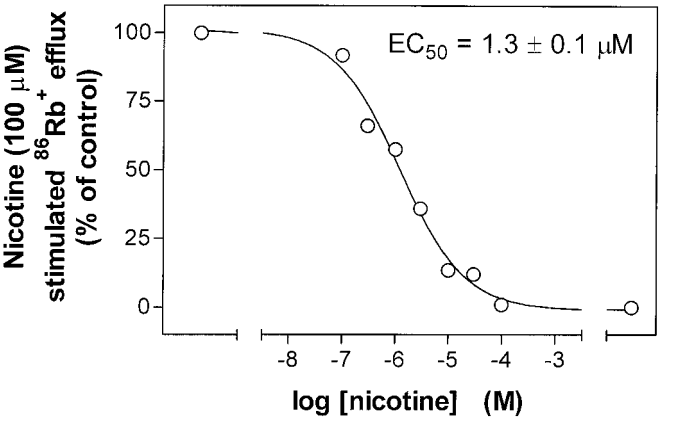


**Fig. 6.** Concentration-dependence for loss of $\alpha3\beta4$ receptor function by treatment with nicotine for 5 days. Cells were grown in culture medium containing the indicated concentration of nicotine for 5 days. They were then loaded with $^{86}$RbCl for 4 h (still in the presence of nicotine) and, after removing the nicotine-containing loading medium, washed three times with nicotine-containing buffer. $^{86}$Rb efflux stimulated by 100 $\mu$M nicotine was then measured as described under *Experimental Procedures*. Results are expressed as a percentage of control samples never exposed to nicotine before the measurement of nicotine-stimulated efflux. Data were fit to an equation for a simple concentration-response relationship. The results shown are from a single representative experiment measured in quadruplicate. This experiment was replicated three times. The $EC_{50}$ value for loss of function by the 5-day treatment with nicotine was 1.3 $\pm$ 0.1 $\mu$M (mean $\pm$ S.E.M., $n = 3$).

function remained at 83 ± 1.2% of that in control cells. The difference in the degree of recovery of function after treatment with 100 μM nicotine for 60 min (93 ± 2%, Table 2) versus 5 days was statistically significant ($P < 0.01$).

**Effects of Chronic Treatments with Nicotine on [$^3$H]EB Binding Sites.** The density of some, but not all, nicotinic receptor subtypes is increased after chronic exposure of rats or mice to nicotine in vivo (Marks et al., 1983; Schwartz and Kellar, 1983; Flores et al., 1992, 1997). Similarly, the density of some nicotinic receptor subtypes expressed in cultured cells is increased by exposure to nicotine for several days, but the degree of increase is subtype-dependent (Peng et al., 1997; Wang et al., 1998; Xiao et al., 2000b). In particular, the β subunit seems to be an important determinant of whether or how much the receptor increases during exposure to nicotine (Wang et al., 1998). To determine whether α3β4 receptors in these cells are increased by exposure to nicotine, [$^3$H]EB binding was measured in cells grown for 5 days in the presence of nicotine at concentrations of 1 to 1000 μM. Binding was measured at a single high concentration of [$^3$H]EB (3 nM), which provides a good estimate of the density of receptor binding sites. As shown in Fig. 8A, continuous exposure of cells to concentrations of nicotine as low as 1 μM for 5 days significantly increased the density of α3β4 receptor binding sites labeled by [$^3$H]EB. The increase was concentration-dependent and ranged from about 200% of control to more than 350% of control. Examination of the time course of changes in [$^3$H]EB binding during incubation with 100 μM nicotine indicated that within 24 h of exposure to nicotine, the number of receptor binding sites was significantly increased to 174% of control and that the receptors continued to increase during the next 4 days of continuous exposure to nicotine, so that after 5 days of exposure to nicotine, the density of receptors had tripled (Fig. 8B).

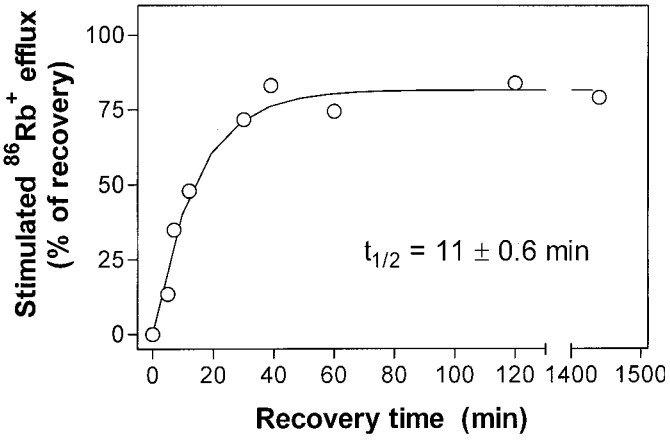


**Fig. 7.** Time course of recovery of α3β4 receptor function after treatment with 100 μM nicotine for 5 days. Cells were grown in culture medium containing 100 μM nicotine for 5 days. They were then loaded with $^{86}$RbCl (still in the presence of nicotine) for 4 h and, after removing the nicotine-containing loading medium, washed three times with buffer containing 100 μM nicotine. $^{86}$Rb efflux stimulated by 100 μM nicotine was then either measured immediately (recovery time 0), or the cells were washed and allowed to recover in fresh medium in the absence of nicotine for the times shown (3 min to up to 24 h) before nicotine-stimulated $^{86}$Rb efflux was measured. Results are expressed as a percentage of recovery of function, which was based on the nicotine-stimulated efflux in parallel control samples incubated for 5 days and prepared in the absence of nicotine. Recovery of receptor function increased with time as a single exponential function described by $Y = Y_{max}(1\text{-}e^{-kt})$. The half-time of the recovery was calculated by $t_{1/2} = 0.693/k$. The results shown are from a single representative experiment measured in quadruplicate. After exposure to 100 μM nicotine for 5 days, function returned to a maximum of 83 ± 1.2% of control function with a $t_{1/2}$ of 11 ± 0.6 min (mean ± S.E.M., $n = 3$).

## Discussion

The KXα3β4R2 cells express a high density of α3β4 nicotinic receptors and provide a good model system for the study of receptor function (Xiao et al., 1998; Zhang et al., 1999). Here we examined the pharmacological profile of α3β4 receptor activation by nicotinic agonists and studied some of the characteristics of receptor desensitization and recovery of function after short-term (up to 60 min) and during long-term (1 to 5 days) exposures to agonists.

The seven nicotinic agonists studied here exhibited a >7000-fold range of potencies in stimulating $^{86}Rb^+$ efflux. EB is by far the most potent of the drugs examined, being 95 times more potent than the second ranked drug, A85380, 500 times more potent than nicotine, and >1000 times more potent than acetylcholine. The rank order of potency is con-

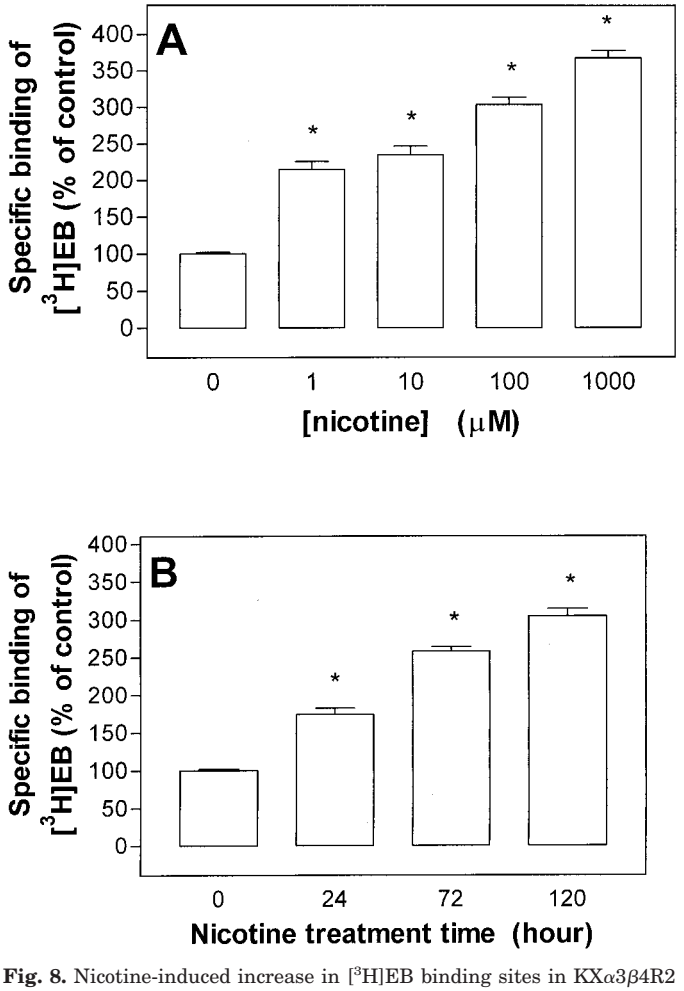


**Fig. 8.** Nicotine-induced increase in [$^3$H]EB binding sites in KXα3β4R2 cell membranes. A, effect of nicotine concentration. Cells were grown in the presence of 1 to 1000 μM nicotine for 5 days before measurement of [$^3$H]EB binding sites. B, time course of nicotine-induced increase in binding sites. Cells were grown in the presence of 100 μM nicotine for 1 to 5 days before measurement of [$^3$H]EB binding sites. Results are expressed as a percentage of control samples grown in parallel in the absence of nicotine. Values are mean ± S.E.M. from three independent measurements. * Values are significantly different from controls ($P <$ 0.01 by one-factor analysis of variance and Dunnett's test).

sistent with that found previously in measurements of whole-cell currents mediated by rat recombinant $\alpha3\beta4$ receptors transiently expressed in HEK 293 cells (Wong et al., 1995), and it is very similar to that found in measurements of whole-cell currents mediated by human $\alpha3\beta4$ receptors expressed in *Xenopus laevis* oocytes (Gerzanich et al., 1997), as well as in measurements of calcium influx mediated by the human $\alpha3\beta4$ receptor expressed in HEK 293 cells (Stauderman et al., 1998).

Compared with nicotine, all of the agonists studied here appeared to be full or nearly full agonists at the $\alpha3\beta4$ receptor, except for DMPP, which produced about 67% of the response elicited by nicotine. This result agrees with a previous study that measured whole-cell currents in cells transiently expressing rat $\alpha3\beta4$ receptors (Wong et al., 1995). However, in studies with human recombinant $\alpha3\beta4$ receptors, DMPP seemed to be as efficacious as nicotine in stimulating calcium influx (Stauderman et al., 1998). Whether this represents a true species difference or a difference attributable to the methods used to assess function is not known. The full agonist activity of cytisine at these $\alpha3\beta4$ receptors agrees with previous measurements in frog oocytes expressing rat $\alpha3\beta4$ receptors (Luetje and Patrick, 1991; Papke and Heinemann, 1994), as well as in mammalian cells transfected with rat (Wong et al., 1995) and human (Stauderman et al., 1998) $\alpha3\beta4$ receptors. Taken together, the pharmacological profile of these recombinant receptors should help to provide a framework useful for identifying $\alpha3\beta4$ receptors in native tissues.

Both nicotine and carbachol desensitize these receptors in a time- and concentration-dependent manner. As would be expected, the half-times for desensitization were inversely related to the concentration of nicotine; thus, at 1 $\mu$M nicotine, the time for half-maximal desensitization was estimated to be about 41 min; at 10 $\mu$M nicotine, it was 11 min; and at 100 $\mu$M nicotine, it was just over 1 min. The $EC_{50}$ values for nicotine and carbachol to desensitize $\alpha3\beta4$ receptors were 3 and 51 $\mu$M, respectively, when the incubation time was 60 min. These concentrations are approximately 10 and 9 times lower, respectively, than the $EC_{50}$ values for activation of the receptor by these agonists. In fact, at the $EC_{50}$ concentrations for desensitization, the $\alpha3\beta4$ receptor would barely be activated ($<$10%, see Fig. 1). Thus, nicotinic agonists are more potent at desensitizing these receptors than at activating them. A similar conclusion has been drawn from previous studies of nicotinic receptors in other systems in vitro (Boyd, 1987; Marks et al., 1994; Rowell and Hillebrand, 1994) and in vivo (Hulihan-Giblin et al., 1990a). However, activation occurs in the millisecond to second time frame, whereas desensitization probably takes place over a longer time frame.

The $\alpha3\beta4$ receptor function began to recover from nicotine-induced desensitization nearly immediately after the cells were washed and placed in recovery buffer. After a 60-min treatment of cells with 300 and 100 $\mu$M nicotine, function recovered to 83 and 93% of control values, respectively. Although in both cases recovery seemed to be nearly complete at about 80 min after placing the cells in nicotine-free medium, the rate of recovery was related to the concentration of nicotine used to induce desensitization. The influence of concentration of agonist on the rate of recovery from desensitization is not immediately predictable from the classic model of desensitization derived from studies of the muscle nicotinic receptor (Katz and Thesleff, 1957), although it is not necessarily irreconcilable with that model. It could, for example, indicate that induction of and recovery from desensitization involve more than one process (Marks et al., 1994; Rowell and Duggan, 1998). To investigate this further, we examined the rate of recovery from carbachol-induced desensitization. Immediately after a 60-min treatment of cells with 1000 or 3000 $\mu$M carbachol, receptor function was decreased by $>$95%, indicating that the receptors were desensitized. As was seen after the 60-min treatment with nicotine, when the cells were washed and placed in recovery buffer, receptor function began to return almost immediately. However, the recovery from carbachol-induced desensitization was not only faster and more complete than that from nicotine but also was independent of the concentration of carbachol used to induce desensitization. This suggests that recovery from carbachol-induced desensitization involves a simple process, rather than a more complex mechanism.

One plausible explanation for the differences in recovery from similar degrees of desensitization induced by nicotine and carbachol is that recovery is related to the agonist's dissociation rate from the receptor. On this basis alone, nicotine, because of its higher affinity, would be expected to have a slower rate of dissociation from the receptor, and receptor recovery would therefore be slower. In addition, nicotine is lipophilic and easily crosses cell membranes, so it may be sequestered inside cells. When the cells are washed and placed in nicotine-free medium, the nicotine inside the cells would be expected to diffuse down its concentration gradient to the extracellular medium, where it could then bind to cell-surface receptors and maintain a certain degree of desensitization. This would, of course, be concentration dependent, and when the extracellular concentration of nicotine dropped below the threshold for receptor desensitization, recovery would be complete. This hypothesis is consistent with a recent observation by Cohen and colleagues who found that nicotine can accumulate in and diffuse from *X. laevis* oocytes at concentrations high enough to desensitize $\alpha4\beta2$ receptors expressed in oocytes not previously exposed to nicotine (B. Cohen, University of California, Riverside, personal communication). This hypothesis is also consistent with a recent finding indicating that changes in sensitivity of nicotinic receptors expressed in *X. laevis* oocytes upon long-term exposure to nicotine result from effects at the extracellular domain of the $\alpha$ subunit (Kuryatov et al., 2000). Carbachol, on the other hand, is a lower affinity, charged quaternary ammonium agonist that does not readily cross cell membranes. Consequently, it is not sequestered inside cells and should be removed rapidly and completely by the washing procedure. According to this explanation, $\alpha3\beta4$ receptor desensitization and recovery of function after exposure to nicotinic agonists for up to 60 min are probably governed by simple processes directly related to agonist occupancy of and removal from the receptor.

There are other possible explanations for the observed differences in recovery of receptor function after exposure to nicotine and carbachol. But if, in fact, nicotine in the brain could be sequestered in and diffuse out of neurons and glial cells, it would have important consequences for its pharmacological actions and the interpretation of its effects. Thus, for example, it could allow nicotine to maintain neuronal

nicotinic receptors in a fully or partially desensitized state for prolonged periods by simple diffusion out of the cell. Moreover, because nicotine's affinity for the α4β2 receptor subtype, which is one of the major nicotinic receptors in brain, is 5- to 8-fold higher than for the α3β4 subtype, desensitization would be expected at nicotine concentrations lower than those seen here.

In addition to desensitization, which occurs and reverses over a time-frame of seconds to minutes, the function of some nicotinic receptors may be inactivated by a much less reversible process, which usually becomes apparent only during longer-term exposure to nicotinic agonists. Inactivation of nicotinic receptors to varying degrees has been found in studies in a variety of tissues, including electric tissue (Aoshima, 1984), neuronal cell lines (Simasko et al., 1986; Boyd, 1987; Lukas, 1991), *X. laevis* oocytes (Kuryatov et al., 2000), and in brain slices and synaptosomes (Marks et al., 1994; Rowell and Duggan, 1998). One in vivo manifestation of receptor inactivation may be the long-term loss (hours to days) of nicotine-induced hormonal responses in rats chronically treated with nicotine (Sharp et al., 1987; Hulihan-Giblin et al., 1990b). The α3β4 receptors studied here recover more than 80% of their function within 2 h after removal from a 5-day continuous exposure to a high concentration of nicotine. Furthermore, the half-time for return of receptor function after this 5-day exposure to nicotine is similar to that after exposure to nicotine for only 60 min (11 min versus 7 min). Based on this, these α3β4 receptors do not seem to undergo extensive inactivation. To the extent that this resistance to inactivation is also a characteristic of native α3β4 receptors, it could have important consequences. For example, even in tissues and CNS areas in which the α3β4 receptor is less dense than other nicotinic receptor subtypes, it may exert a disproportionate influence on cell function if the other subtypes undergo extensive inactivation.

There was, however, a 17% loss of receptor function that seemed to be irreversible within the 24-h recovery period that was examined. Whether this irreversible loss of receptor function reflects the process of inactivation, a shift in the equilibrium between the resting (activatable) conformation of the receptor and the desensitized conformation, or a cellular process such as receptor internalization is not known.

The number of α3β4 receptors in these cells was increased by exposure to nicotine in a time- and concentration-dependent manner; thus, receptor binding sites doubled after a 5-day exposure to 1 μM nicotine and were increased by more than 3-fold in cells exposed to higher concentrations of nicotine. This increase in α3β4 receptor binding sites contrasts with a recent report that found that human α3β4 nicotinic receptors expressed in cells derived from the HEK 293 cell line were not significantly increased by exposure to nicotine for up to 48 h (Wang et al., 1998). There are certain methodological differences between the two studies, such as binding to total cell membrane preparations versus binding to soluble nAChRs, but we cannot exclude the possibility of a species difference between rat and human receptor. However, we believe a likely explanation for the difference is that the nicotine-induced increase of receptors in the cells used here reflects a faster rate of receptor turnover.

The β subunit seems to be an important determinant of the degree of up-regulation of the nicotinic receptor binding site. For example, in transfected cells, receptors containing β2 subunits are, in general, increased much more than those containing β4 subunits (Wang et al., 1998; Xiao et al., 2000b). This may reflect a more pronounced effect of nicotine on the assembly and/or turnover of β2-containing receptors than on β4-containing receptors (Wang et al., 1998). Although it is difficult to make a precise comparison between receptors in transfected cells and native receptors in mammalian nervous system, it is notable that whereas α4β2 nicotinic receptors in rat brain are increased by chronic administration of nicotine (Flores et al., 1992), the nicotinic receptors in adrenal gland, superior cervical ganglia, and pineal gland, which seem to be predominantly α3β4 receptors, are not increased (Flores et al., 1997; Dávila-García and Kellar, 1998). One explanation for the failure of chronic nicotine administration to increase neuronal nicotinic receptors in these peripheral tissues is that the increase may be directly related to the turnover rate of the receptors. Thus, if the receptor turnover rate in peripheral neurons was low compared with the rate in these cells, there would be a proportionately smaller increase in the receptor density over the time period of chronic administration.

The increased density of receptors found in the studies here did not lead to a measurable increase in receptor function in the cells. Previous studies demonstrated that [$^3$H]EB binds not in cells expressing either α3 or β4 subunits alone, only in cells expressing both subunits (Xiao et al., 1998). Furthermore, although expression of both subunits in a single binding entity is the minimum requirement for endowing the cell with functional nicotinic receptors, it does not assure function. Thus, although the α3β4 receptors normally expressed in these cells function at a high level (Xiao et al., 1998; Zhang et al., 1999), it is possible that the additional [$^3$H]EB binding sites reflect a pool of assembled receptors that are no longer, or possibly never were, on the cell surface. Such a nicotine-induced increase in an intracellular pool of receptors in cells expressing α4β2 receptors has been reported (Whiteaker et al., 1998). If the differences between the functional and nonfunctional pools of nicotinic receptors are better understood, it could open the possibility to increasing nicotinic receptor function in conditions thought to involve these receptors, including Alzheimer's disease, Tourette's syndrome, attention deficit disorder, and nicotine addiction.

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**Address correspondence to:** Kenneth J. Kellar, Department of Pharmacology, Georgetown University School of Medicine, Washington, DC 20007. E-mail: kellark@georgetown.edu