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Nicotine-Induced Increase in Neuronal Nicotinic Receptors Results from a Decrease in the Rate of Receptor Turnover

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SUMMARY

Chronic nicotine exposure in tobacco smokers or experimental animals is known to cause an increase in brain binding sites for nicotine. It has been proposed that this is an adaptive response of neurons to accumulation of chronically desensitized receptors. Acetylcholine receptors of the same $(\alpha4)_2(\beta2)_3$ subunit composition as the predominant subtype of brain nicotinic receptors with high affinity for nicotine have been expressed in *Xenopus* oocytes and in a permanently transfected fibroblast cell line. Chronic exposure of these cells to nicotine or another agonist is shown to result in an increase in receptor amount, indicating that nicotine-induced up-regulation reflects properties of the $\alpha4\beta2$ receptor protein, rather than being an adaptive response unique to the neurons in which these receptors are normally expressed. The nicotine concentration dependence, time course, and extent of receptor up-regulation are similar to those reported for receptors in brain. Up-regulation does not appear to require ion flow through the ion channel, because it is also caused by mecamylamine, which blocks the ion channel, and because after prolonged exposure to nicotine most receptors become permanently unable to open their channels in response to nicotine binding. The noncompetetive antagonist mecamylamine blocks open channels more effectively, and so it is more effective at blocking channels in the presence of nicotine. Mecamylamine and nicotine are also synergistic in causing receptor up-regulation. Ligands that cause up-regulation appear to induce a conformation of the receptor that is removed from the surface and degraded more slowly.

Addiction to tobacco, which is predicted to cause 250 million premature deaths worldwide by the turn of the century (1), is thought to be caused by nicotine acting on AChRs in the brain (2). Up-regulation of brain AChRs is a characteristic, but puzzling, feature of addiction to nicotine (3). The number of high affinity nicotine binding sites in the brains of tobacco smokers and animals chronically given nicotine is increased up to 2-fold (3–7). It has been suggested that the increase in AChRs in response to chronic exposure to agonist during the development of tolerance is an adaptive response of neurons to inactivation of AChRs by desensitization (3).

The predominant subtype of brain AChR with high affinity for nicotine is formed from $\alpha4$ and $\beta2$ subunits (8) and has the subunit stoichiometry $(\alpha4)_2(\beta2)_3$ (9, 10). This subtype is up-regulated in amount after chronic treatment of rats with nicotine (6). This up-regulation does not appear to be accompanied by an increase in brain $\alpha4$ or $\beta2$ mRNA (7).

The $\alpha4$ and $\beta2$ subunits of chicken neuronal AChRs have been permanently transfected into mouse L cell fibroblasts under the control of a dexamethasone-inducible promoter to produce the cell line M10 (11). $\alpha4\beta2$ AChRs in M10 cells exhibit the biochemical, electrophysiological, and pharmacological properties expected of native brain $\alpha4\beta2$ AChRs (11, 12). $\alpha4\beta2$ AChRs can also be expressed in *Xenopus* oocytes injected with $\alpha4$ and $\beta2$ mRNAs and in this transiently expressed state are especially convenient for electrophysiological studies (13, 14).

Here we use M10 cells and *Xenopus* oocytes injected with $\alpha4$ and $\beta2$ mRNAs to demonstrate that nicotine-induced up-regulation of $\alpha4\beta2$ AChRs is an intrinsic property of this protein, rather than an adaptive process characteristic of the neurons in which these AChRs are normally expressed. It is demonstrated that nicotine-induced up-regulation of $\alpha4\beta2$ AChRs results from a decrease in the rate of AChR turnover. Up-regulation does not appear to require a signal transmitted by ion flow through the AChR channel but instead appears to result from a change in AChR conformation associated with a long-lasting inactive state.

Materials and Methods

M10 cells. This line of mouse fibroblasts transfected with chicken brain AChR $\alpha4$ and $\beta2$ subunits has been described previously (11).

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ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; mAb, monoclonal antibody; DMPP, 1,1-dimethyl-4-phenylpiperazinium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; SSPE, standard saline/phosphate/EDTA.

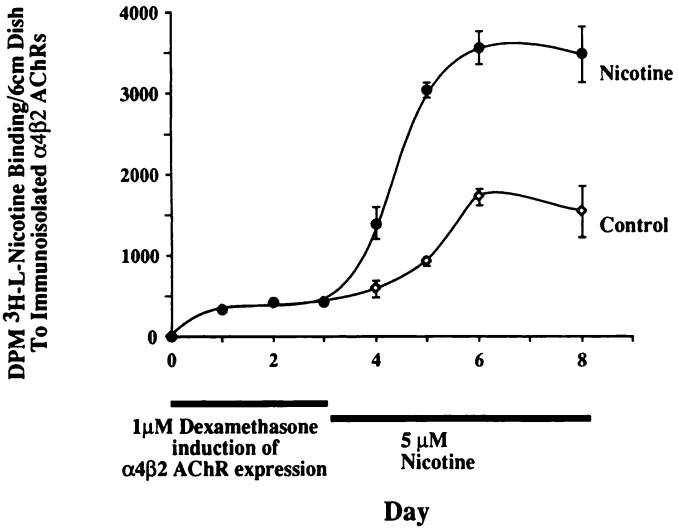

Fig. 1. Time dependence of nicotine-induced up-regulation. [^{3}H]Nicotine labeling of immunoisolated $\alpha4\beta2$ AChRs was quantitated in extracts of duplicate M10 cultures at time points during induction with dexamethasone and after subsequent exposure to fresh medium with or without nicotine.

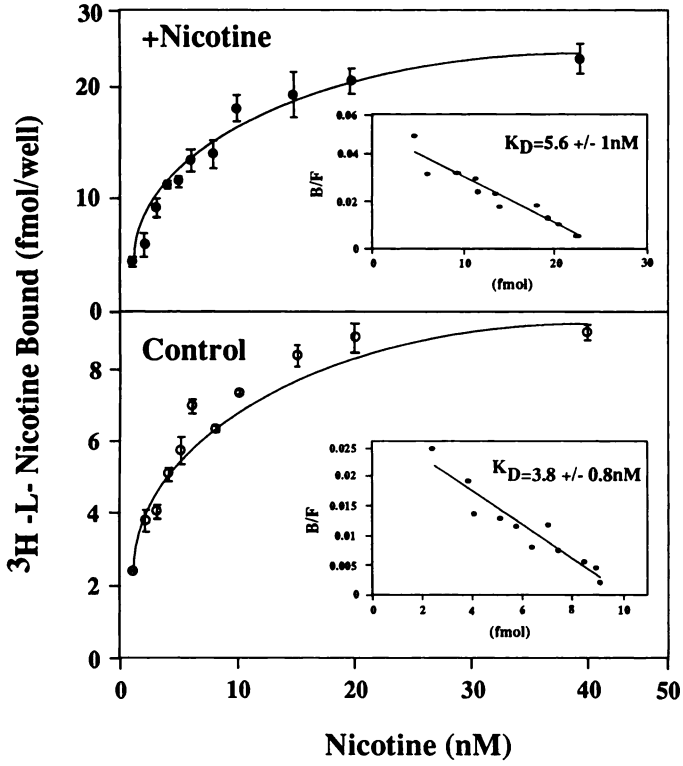

Fig. 2. Evidence that nicotine-induced up-regulation does not alter the affinity of $\alpha4\beta2$ AChRs for nicotine. Control dexamethasone-induced M10 cells or parallel cultures were exposed to 5 μM nicotine for 3 days. AChRs were solubilized with Triton X-100, immunoisolated on microwells coated with a mAb to $\beta2$ subunits, and then assayed for binding with various concentrations of L-[^{3}H]nicotine. Scatchard plots (*insets*) revealed a 2.55-fold increase in nicotine binding sites in the nicotine-treated cells, with no significant change in binding affinity (K_d of 3.8 ± 0.8 nM in control versus 5.6 ± 1.0 nM in nicotine-treated cultures).

***Xenopus* oocytes.** Oocytes were prepared for microinjection by standard techniques and injected with 15 ng each of cRNAs for chicken $\alpha4$ and $\beta2$ subunits. Oocytes were kept semi-sterily in 50% L-15 medium (GIBCO-BRL) with 10 mM HEPES buffer, pH 7.5.

mAbs. mAb 290 to $\beta2$ subunits and mAb 299 to $\alpha4$ subunits were raised to immunoaffinity-purified rat brain $\alpha4\beta2$ AChRs as described previously (8).

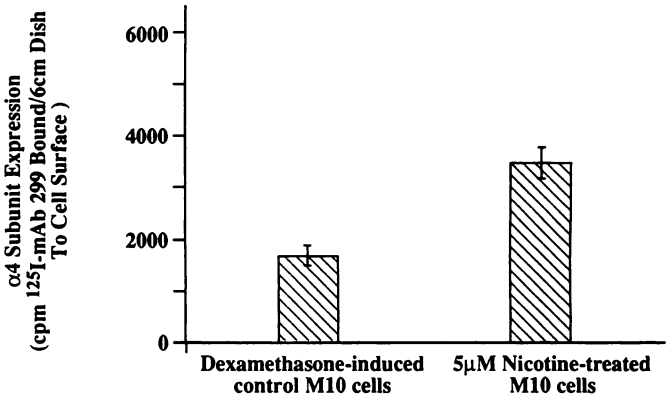

Fig. 3. Quantitation of $\alpha4\beta2$ AChRs by binding of an ^{125}I-labeled mAb to $\alpha4$. Dexamethasone-induced M10 cells were incubated with or without nicotine for 3 days; $\alpha4\beta2$ AChRs on the cell surface were measured by binding to intact cells of an ^{125}I-labeled mAb to $\alpha4$.

Assays of $\alpha4\beta2$ AChRs. Binding of ^{125}I-mAb 299 to the surface of intact M10 cells was measured in six-well dishes using 2 nM ^{125}I-mAb 299 (specific activity, 3.21 × 10^{18} cpm/mol), in 1 ml of medium/well, for 4 hr at 37°. Nonspecific binding was measured using uninduced M10 cells.

Cultures of M10 cells were solubilized in 400 μl of 2% Triton X-100, 50 mM NaCl, 50 mM sodium phosphate buffer, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 5 mM iodoacetamide. Particulate material was then pelleted by centrifugation in a microfuge at 4°, and the supernatant was used for assays.

$\alpha4\beta2$ AChRs were immunoisolated from detergent extracts using Immulon 4 microwells (Dynatech) that had been coated with mAb 290 by overnight incubation with 100 μl of 40 μg/ml Protein G-purified IgG in 10 mM bicarbonate buffer, pH 8.8, followed by blockage with 250 μl of 3% bovine serum albumin in 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, 0.05% Tween 20, for 2 hr at room temperature and three washes with 200 μl of 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, 0.5% Triton X-100. Aliquots of 100 μl of $\alpha4\beta2$ AChR extract were allowed to bind overnight at 4°. After rinsing three times, 100 μl of 20 nM L-[^{3}H]nicotine (72 Ci/mmol; Amersham) in 0.5% Triton X-100, 100 mM NaCl, 10 mM sodium phosphate buffer, pH 7.5, were added for 40 min at 4°. After three rapid washes, bound [^{3}H]nicotine was removed using 100 μl of 2.5% SDS, 5% 2-mercaptoethanol, for 30 min and was measured using a scintillation counter. Nonspecific binding was measured using blocked wells lacking mAb.

$\alpha4\beta2$ AChRs were also quantitated by [^{3}H]nicotine binding using a method in which the detergent-solubilized AChRs were immobilized on 0.3% polyethyleneimine-coated Whatman GF/B glass fiber filters instead of by a subunit-specific mAb attached to a microwell. This method was basically as described previously for brain extract (15, 16). Triplicate 100-μl aliquots of M10 cell extracts were incubated with 20 nM [^{3}H]nicotine for 20 min at room temperature, diluted with 4 ml of ice-cold 50 mM Tris, pH 7.4, and then filtered. After rapid washing three times with 4 ml of buffer, bound [^{3}H]nicotine was measured by scintillation counting. Nonspecific binding was determined using uninduced M10 cells.

Northern analysis. Total RNA was isolated, by the method of Chomczynski and Sacchi (17), from induced M10 cells that had been treated with or without 5 μM nicotine for 3 days. Aliquots of 25 μg of RNA were electrophoresed for 16 hr at 24 V in 1% agarose gels containing 1.1 M formaldehyde, 0.02 M 3-(*N*-morpholino)ethanesulfonic acid, 0.05 M sodium acetate buffer, pH 8.0, and 0.01 M EDTA. The gels were rinsed in water treated with 0.1% (v/v) diethylpyrocarbonate and were then soaked in 10× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4, 1 mM EDTA) for 45 min. RNA was vacuum transferred to a Nytran membrane (Schleicher

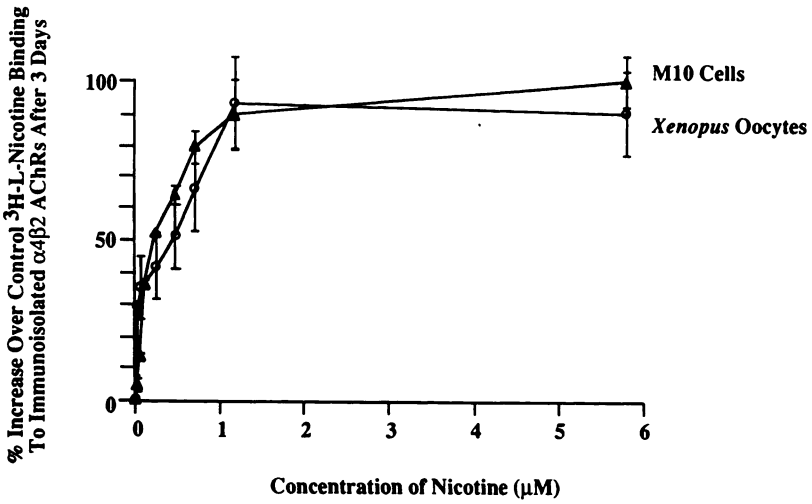

Fig. 4. Dose dependence of nicotine-induced up-regulation. Duplicate M10 cultures were grown to confluence before expression of $\alpha4\beta2$ AChRs was induced by exposure to 1 μM dexamethasone for 3 days. Fresh medium with or without nicotine was added for an additional 3 days. Finally, $\alpha4\beta2$ AChRs were solubilized using Triton X-100, immunoisolated on microwells coated with a mAb to $\beta2$ subunits, and quantitated by labeling with [^{3}H]nicotine. *Xenopus* oocytes injected with 15 ng of cRNAs for $\alpha4$ and $\beta2$ subunits were incubated for 3 days to allow expression of AChR protein and were then incubated for an additional 3 days with the indicated concentrations of nicotine before solid-phase radioimmunoassay. Each *point* is the mean of five oocytes.

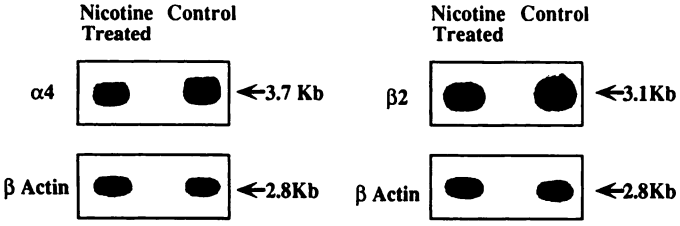

Fig. 5. Amounts of $\alpha4$ and $\beta2$ subunits mRNA measured using Northern blots. Results of one of two experiments are shown. A series of exposures ensured that measurements were made in the linear range. Quantitation by scanning of the autoradiographs and normalization to β-actin controls averaged over two experiments revealed ratios of nicotine-treated to control values of 0.85 for $\alpha4$ and 0.86 for $\beta2$.

and Schuell) and UV cross-linked. Hybridization was performed overnight at 42°, using a random-primed, [^{32}P]dATP-labeled, chick $\alpha4$ or $\beta2$ subunit cDNA fragment or a rat β-actin cDNA fragment, in 40% formamide, 5× Denhardt's solution, 0.5% SDS, 5× SSPE, 0.15 mg/ml denatured salmon sperm DNA. Rat β-actin was used as a heterologous probe to determine the amount of mouse β-actin RNA constitutively expressed in L cells, thereby allowing for normalization of the $\alpha4$ and $\beta2$ mRNA signals within each lane. Membranes were washed at 50° in 1× SSPE/0.1% SDS before autoradiography at −70°, using Kodak XAR-5 film. Multiple exposures ensured that the film was exposed in the linear range. Scanned images were quantitated using Image 1.54 software (National Institutes of Health).

Electrophysiological recordings. Currents from M10 cells were recorded using a whole-cell configuration, as described previously (11). M10 cells were subcultured onto uncoated glass coverslips for 3–5 days after plating and induction with 1 μM dexamethasone. The coverslips were placed in a recording chamber, which was continuously perfused with 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES buffer, pH 7.4, 11 mM glucose. Patch pipettes (2–4 MΩ) were filled with 140 mM cesium gluconate, 20 mM NaCl, 2 mM $CaCl_2$, 5 mM HEPES buffer, pH 7.4, 10 mM EGTA. Recordings used an Axopatch-1D amplifier (Axon Instruments). Drugs were applied by gravity perfusion from a set of eight glass tubes (300-μm diameter) positioned manually using a hydraulic micromanipulator (Narishige). Records were digitized (using a Labmaster DMA; Axon Instruments) and stored on a Gateway PC486 computer.

Currents from *Xenopus* oocytes injected with $\alpha4$ and $\beta2$ cRNAs were measured by a conventional two-electrode voltage-clamp method (using an OC-725 amplifier; Warner Instrument Corp.), as described previously (18). The recording chamber was continuously perfused with 96 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6. Drugs were applied by gravity flow through a set of seven glass tubes (2-mm diameter) positioned using a manual manipulator (Brinkman). Data were digitized using MacLab software and hardware (AD

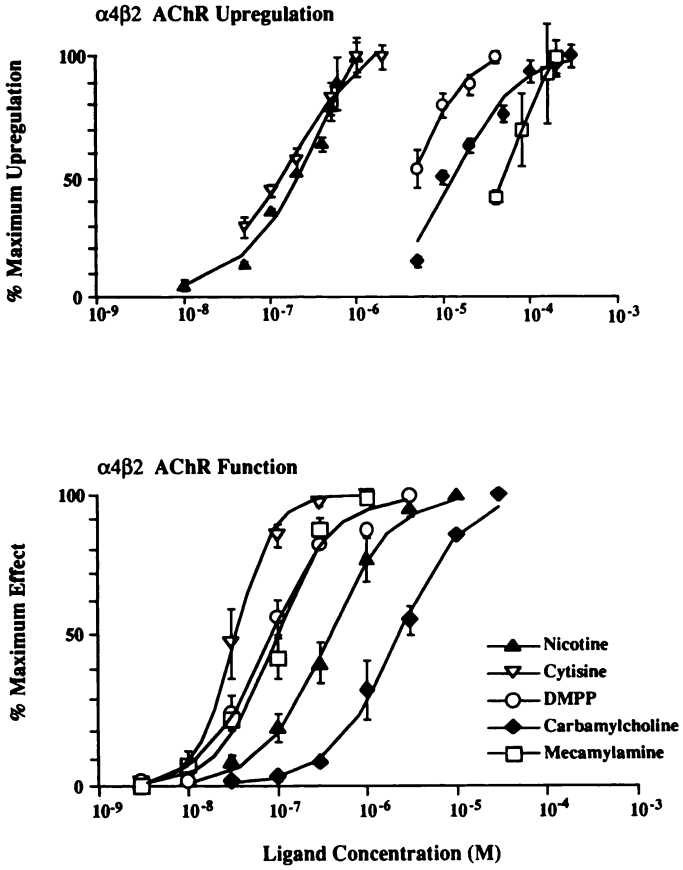

Fig. 6. Comparison of dose-response relationships for $\alpha4\beta2$ AChR up-regulation in M10 cells and function in *Xenopus* oocytes. M10 cells were grown to confluence in 6-cm dishes, induced for 3 days with 1 μM dexamethasone, treated for 3 days with the indicated ligands, and then assayed for [^{3}H]nicotine binding to AChRs immunoisolated through their $\beta2$ subunit. Each *point* is the mean of three dishes. Function was assessed by voltage-clamp measurements in *Xenopus* oocytes 3 days after injection of 15 ng each of $\alpha4$ and $\beta2$ cRNAs. Each *point* is the average of three to five oocytes. Data were normalized to maximum current with saturating agonist concentrations. All agonists tested were full agonists. Blockage by mecamylamine was measured with 1 μM nicotine. Data were fit by the Hill equation.

TABLE 1
Pharmacological properties of $\alpha4\beta2$ AChRs

	Up-regulation, EC_{50}[a]	Function, EC_{50}[a]		Equilibrium binding, K_i
		Activation	Blocking	
	μM	μM		μM
Agonists				
Cytisine	0.17 ± 0.02	0.031 ± 0.003		0.00014 ± 0.00003[b]
Nicotine	0.21 ± 0.04	0.35 ± 0.02		0.0039 ± 0.00021[b]
DMPP	3.0 ± 0.3	0.073 ± 0.01		0.0094 ± 0.0002
Carbamylcholine	15 ± 4	2.5 ± 0.3		0.36 ± 0.013[b]
Competitive antagonist				
Curare	None		2.7 ± 0.5	25.0 ± 14[b]
Noncompetitive antagonist				
Mecamylamine	65 ± 6		0.37 ± 0.5	>1000[b]

[a] Data from Fig. 6.
[b] Data from Ref. 11.

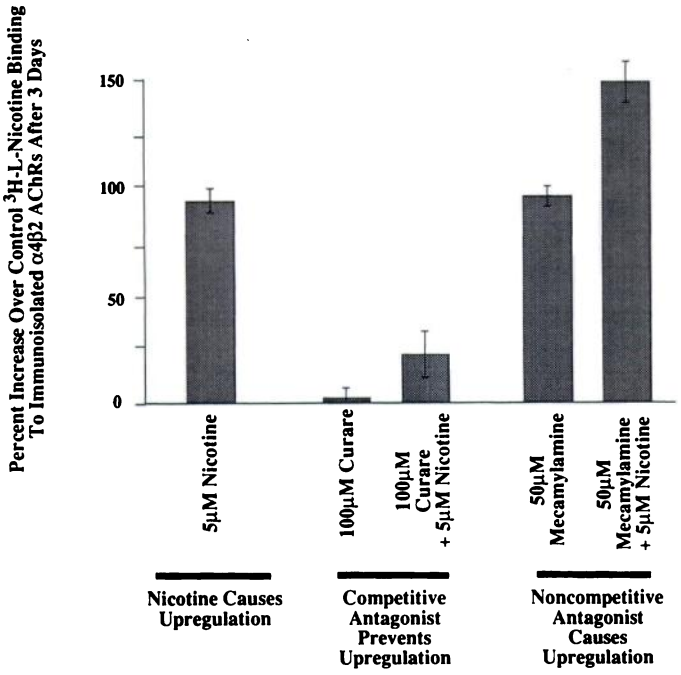

Fig. 7. Effects of antagonists on up-regulation of $\alpha4\beta2$ AChRs in M10 cells. M10 cells were grown to confluence in 6-cm dishes, induced for 3 days with 1 μM dexamethasone, and then treated for 3 days with the indicated ligands before solid-phase radioimmunoassay. Each *bar* is the mean value of three dishes.

Instruments) and were stored on an Apple MacIntosh IIcx computer. Data obtained from patch-clamp and two-electrode voltage-clamp recordings were analyzed using pCLAMP and AxoGraph software (Axon Instruments).

Results and Discussion

Chronic exposure of dexamethasone-induced M10 cells to nicotine causes an increase in the amount of AChRs, which reaches a maximum of 2 times control in 3 days (Fig. 1). There is no increase in cell number, compared with control cultures. The extent and time course of the up-regulation of $\alpha4\beta2$ AChRs in M10 cells are comparable to the 2-fold increase over 4 days observed for nicotine binding sites in brains of mice chronically treated with nicotine (19).

The increase in [^{3}H]nicotine binding to immunoisolated AChRs results from an increase in the amount of AChR, rather than from an increase in the affinity of the AChR for nicotine. This was shown by Scatchard plots of [^{3}H]nicotine binding to control and chronically nicotine-treated M10 cells (Fig. 2). As an additional control to show that after chronic exposure to nicotine the AChRs do not change in their affinity for the subunit-specific mAb used to immunoisolate them and the change in nicotine binding is not somehow induced by mAb binding, [^{3}H]nicotine binding was also measured in an assay in which solubilized AChRs were isolated on polyethylenimine-coated glass fiber filters instead of by a mAb attached to a microwell. In this assay also, 3-day exposure of M10 cells to 5 μM nicotine increases the number of [^{3}H]nicotine binding sites by 2.5-fold.

Up-regulation in $\alpha4\beta2$ AChR expression can also be measured as an increase in the amount of $\alpha4\beta2$ AChR protein detected on the cell surface by binding of an ^{125}I-labeled mAb to $\alpha4$ subunits (Fig. 3). This clearly confirms that the increase in [^{3}H]nicotine binding to detergent-solubilized and immunoisolated AChRs observed in Figs. 1 and 2 results from an increase in the amount of $\alpha4\beta2$ AChR protein, rather than

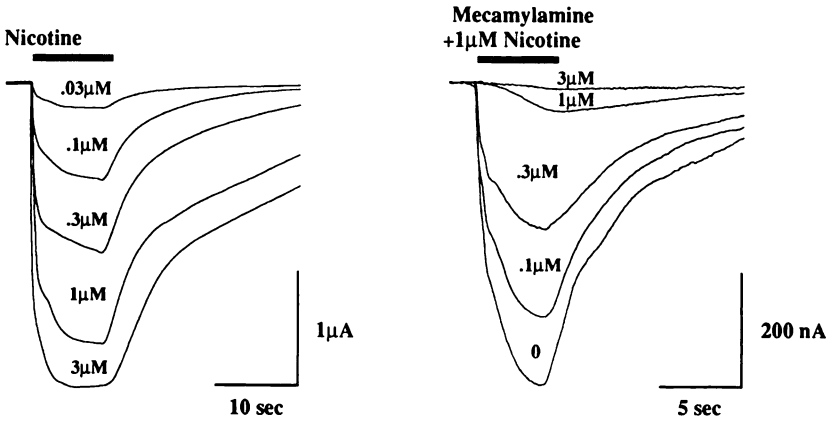

Fig. 8. Efficient blockage by mecamylamine of the response to nicotine of $\alpha4\beta2$ AChRs expressed in *Xenopus* oocytes. *Left*, responses to various concentrations of nicotine; *right*, blockage of the response to 1 μM nicotine by successively increasing concentrations of mecamylamine applied together with nicotine in another oocyte. This illustrates primary data of the type used to plot Fig. 6 and provide data for Table 1.

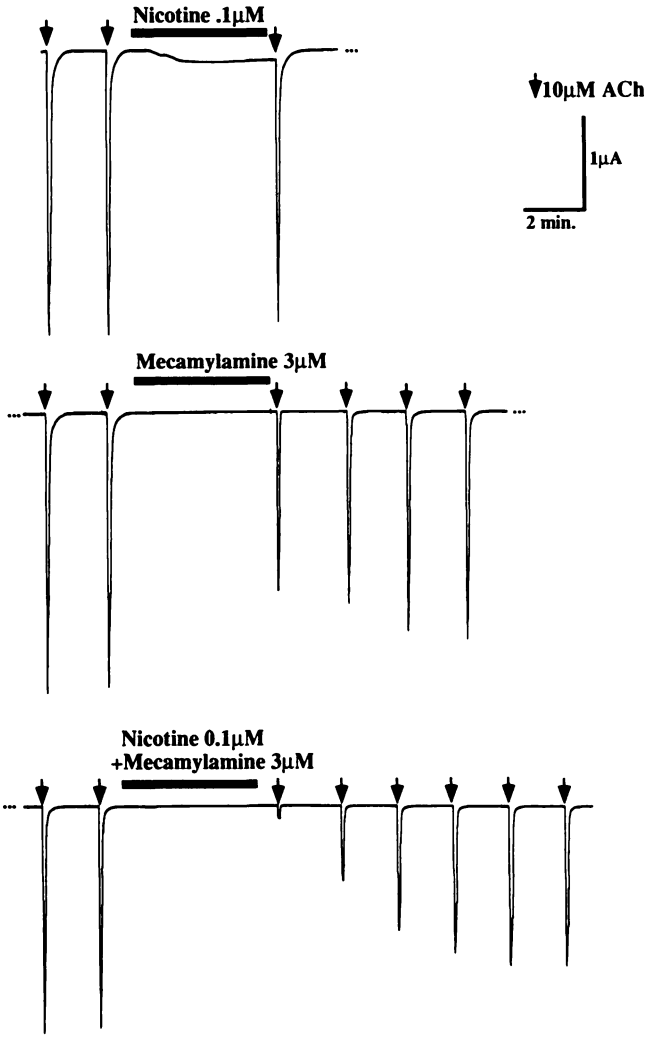

Fig. 9. Evidence that mecamylamine blockage of $\alpha4\beta2$ AChR function is more effective in the presence of nicotine. A continuous series of responses to a saturating concentration of ACh is shown from *top* to *bottom*. *Top*, when a high concentration of ACh (10 μM = 21 × its EC_{50}) was applied for 5 sec and then washed off over 2 min, the same response could be obtained again. Then a low concentration of nicotine (0.1 μM = 0.3 × its EC_{50}) applied for a longer time (5 min) caused only a small current (5% of maximum) and caused little accumulated desensitization of a response to 10 μM ACh applied immediately afterwards. *Middle*, two control responses to 10 μM ACh followed by 5 min of exposure to 3 μM mecamylamine are shown. Mecamylamine caused no current and partially (36%) blocked the response to ACh immediately thereafter. There was some additional recovery in subsequent responses. *Bottom*, when mecamylamine was applied with nicotine the extent of blockage by mecamylamine was 2.6-fold greater and nearly complete (94%). There was some recovery on washing, but after 10 min of washing the response was still blocked by 28%, raising the possibility that long term blockage was caused by mecamylamine trapped in channels.

from an increase in the binding affinity for nicotine. This also indicates that $\alpha4\beta2$ AChRs are up-regulated on the cell surface and not just in an intracellular pool.

Nicotine-induced up-regulation of $\alpha4\beta2$ AChRs expressed in both mouse fibroblasts and *Xenopus* oocytes proves that up-regulation does not depend on mechanisms unique to neurons (Fig. 4). The concentration dependence of nicotine-induced up-regulation reveals a half-maximally effective concentration (EC_{50}) in M10 cells of 2.1×10^{-7} M nicotine (Fig. 4). Nicotine also induces up-regulation of $\alpha4\beta2$ AChRs expressed in *Xenopus* oocytes with a similar concentration dependence (EC_{50} = 1.9×10^{-7} M) (Fig. 4). The EC_{50} for nicotine-induced up-regulation of $\alpha4\beta2$ AChRs (2×10^{-7} M) is a physiologically significant concentration very close to the typical, mean, steady state, serum concentration of nicotine in smokers (1.5×10^{-7} M) (20).

Nicotine treatment does not up-regulate the steady state amounts of mRNA for $\alpha4$ or $\beta2$ subunits in M10 cells (Fig. 5). The average values from two independent experiments reveal ratios of nicotine-treated to control values of 0.85 ± 0.01 for $\alpha4$ mRNA and 0.86 ± 0.01 for $\beta2$ mRNA. Nicotine-induced up-regulation of $\alpha4\beta2$ AChRs in cells transfected using cDNAs that lack AChR-specific promoters and lack of up-regulation of $\alpha4$ and $\beta2$ mRNA by nicotine in the transfected cells indicate that up-regulation is independent of transcriptional events. The failure of nicotine to up-regulate transcription of $\alpha4$ or $\beta2$ subunit mRNA while up-regulating the amount of $\alpha4\beta2$ AChR protein in fibroblasts transfected with $\alpha4$ and $\beta2$ subunits is consistent with similar results observed in brain (6, 7) and suggests that nicotine up-regulates $\alpha4\beta2$ AChRs by a post-transcriptional mechanism. The conclusion is further confirmed by the observation that nicotine-induced up-regulation of $\alpha4\beta2$ AChRs also occurs in *Xenopus* oocytes when fixed amounts of cRNA for each subunit are injected into the oocytes (Fig. 4).

Other agonists besides nicotine are also capable of up-regulating $\alpha4\beta2$ AChRs in M10 cells (Fig. 6; Table 1). Nicotine and cytisine are able to efficiently cross cell membranes because they are tertiary amines; thus, their ability to up-regulate $\alpha4\beta2$ AChRs might, in principle, be due to an intracellular action promoting conformational maturation of $\alpha4$ subunits or assembly of $\alpha4$ with $\beta2$ subunits. However, because the membrane-impermeable, quaternary amine, agonists DMPP and carbamylcholine also cause up-regulation, these agonists may be assumed to produce their effects from outside the cell, on $\alpha4\beta2$ AChRs that are already in the surface membrane.

Ion flow through activated $\alpha4\beta2$ AChRs does not appear to be necessary to induce up-regulation. As expected, the competitive antagonist curare blocks up-regulation by agonists by preventing their binding to the ACh binding site (Fig. 7; Table 1). However, mecamylamine is an antagonist (Figs. 6 and 8; Table 1) but causes up-regulation and is partially additive with nicotine in its up-regulation effects (Fig. 7). Similar, partially additive effects of these ligands have been reported for up-regulation of [^{3}H]nicotine binding in rat brains (21). Some noncompetitive inhibitors block open channels and are more effective in the presence of agonists (22). If mecamylamine behaved in this way, then this would explain its synergistic effect with nicotine. Mecamylamine is a noncompetitive antagonist that blocks the cation channel (23, 24), as shown by its efficient blockage of the response to nicotine (Figs. 6 and 8; Table 1) but very inefficient blockage of [^{3}H]nicotine binding (11) (Table 1). The synergistic effect of nicotine and mecamylamine in blocking AChR function shown in Fig. 9 is consistent with the idea that mecamylamine is an open channel blocker. Fig. 9 shows that mecamylamine is more effective in blocking the function of $\alpha4\beta2$ AChRs when applied in the presence of a low concentration of nicotine. The synergism between nicotine and mecamylamine in up-regulating $\alpha4\beta2$ AChR amounts (Fig. 7) may also result from a process in which nicotine activates AChRs, allowing mecamylamine to bind more effectively to the AChR and keep it in a conformation that cannot pass ions but that results in up-regulation. Additional evidence that ion flow

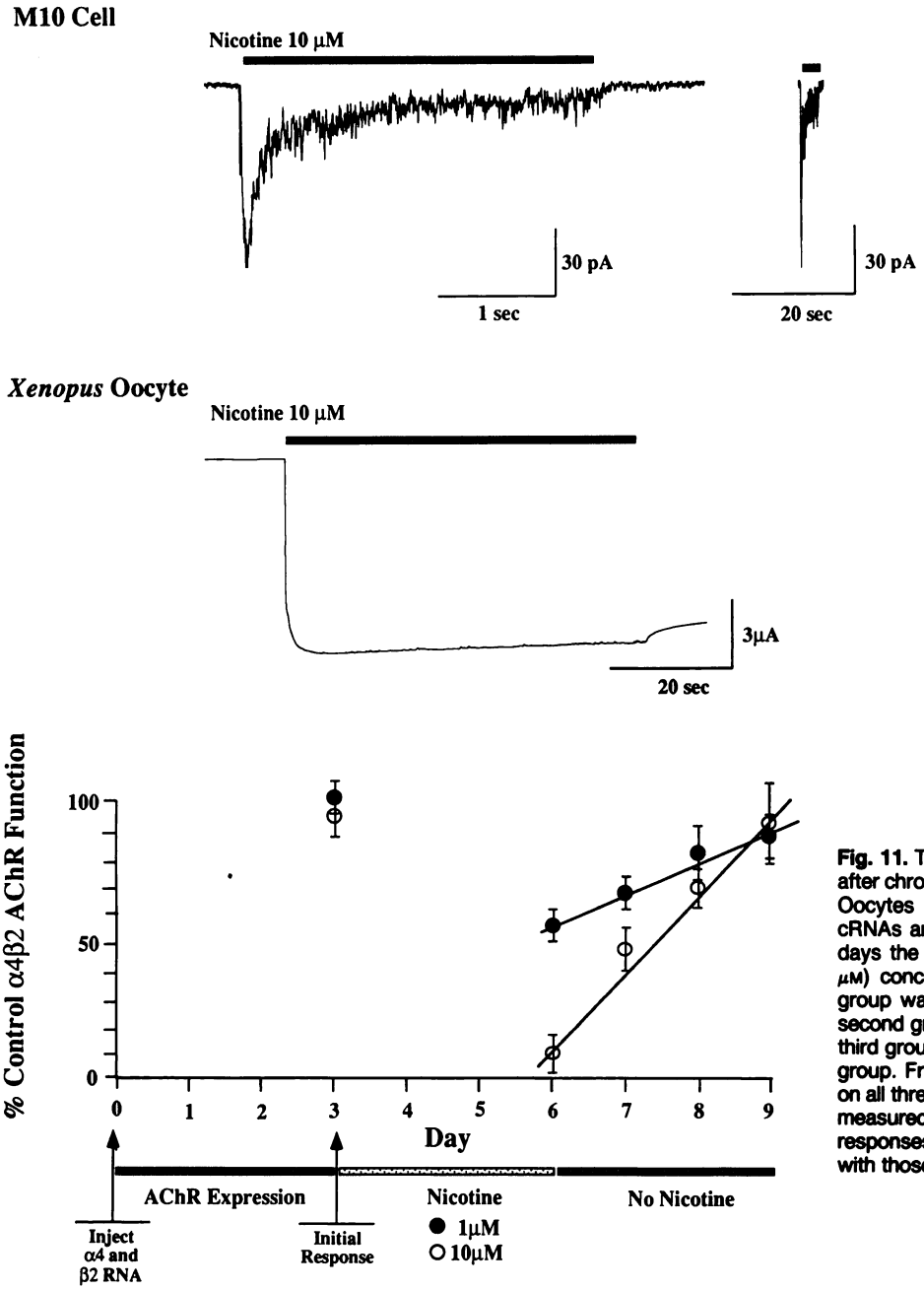

Fig. 10. More rapid desensitization of $\alpha4\beta2$ AChRs in transfected mouse fibroblasts (*top*) than in *Xenopus* oocytes (*bottom*). *Top*, *left*, nicotine-induced whole-cell current from an M10 cell 4 days after induction with dexamethasone; *right*, data plotted on the same scale used to show the response in a *Xenopus* oocyte (*bottom*), to emphasize how much more rapidly the $\alpha4\beta2$ AChRs in M10 cells desensitize, compared with those in oocytes. The reason for this difference in desensitization rate is not known, although it could perhaps result from differences in phosphorylation in the two cell types, because phosphorylation is known to influence the rate of AChR desensitization (30).

Fig. 11. Time course of recovery of $\alpha4\beta2$ AChR function after chronic treatment of *Xenopus* oocytes with nicotine. Oocytes were injected with 12 ng each of $\alpha4$ and $\beta2$ cRNAs and divided into three groups of seven. After 3 days the responses of all oocytes to a saturating (100 μM) concentration of ACh were measured. Then one group was incubated for 3 days with 1 μM nicotine, a second group was incubated with 10 μM nicotine, and a third group was incubated without nicotine, as a control group. Fresh medium lacking nicotine was then placed on all three groups and the response to 100 μM ACh was measured repeatedly over the next 3 days. Each day the responses of each group were averaged and compared with those of the control group.

through the AChRs is not required for up-regulation is provided by the observation that $\alpha4\beta2$ AChRs are desensitized much more rapidly when expressed in M10 cells than when expressed in *Xenopus* oocytes (Fig. 10) but up-regulation occurs equally effectively in the two cases (Fig. 4). In fact, in *Xenopus* oocytes even after chronic exposure to nicotine for 3 days most of the AChRs are in a conformation that can bind nicotine (Fig. 4) but that cannot respond by opening the cation channel (Fig. 11). This conformation differs from the short term desensitized conformation, which recovers over seconds or minutes, because after 3 days of exposure to nicotine another 3 days are required to fully recover activity. This slow rate of recovery resembles the rate of AChR synthesis in *Xenopus* oocytes (25), rather than the rate of recovery from short term desensitization.

Others have also observed permanently desensitized AChRs after chronic exposure to nicotine (26–28) and nicotine-induced up-regulation of functionally blocked AChRs (29), both in cultured cells and in brains of intact animals. An irreversibly desensitized state similar to that which we observed for $\alpha4\beta2$ AChRs expressed in *Xenopus* oocytes (Fig. 11) was reported after chronic agonist exposure of $\alpha3$ AChRs in PC-12 pheochromocytoma cells (26). Chronic exposure of mice to nicotine has been shown to result in a dose-dependent decrease in AChR function, measured both behaviorally as tolerence to nicotine-induced decreases in body temperature and locomotor activity and biochemically as a decrease in nicotine-induced [^{3}H]dopamine release from striatal synaptosomes (27). Recovery of AChR function *in vivo* after chronic nicotine treatment has been shown to require several days (27, 28), e.g., for the recovery of the effect of nicotine on prolactin release to return to normal (28). This is similar to the rate of recovery of function that we observed in *Xenopus* oocytes (Fig. 11). Evidence that $\alpha4\beta2$ AChR function in brain also is not necessary for nicotine-induced up-regulation is provided by the demonstration that

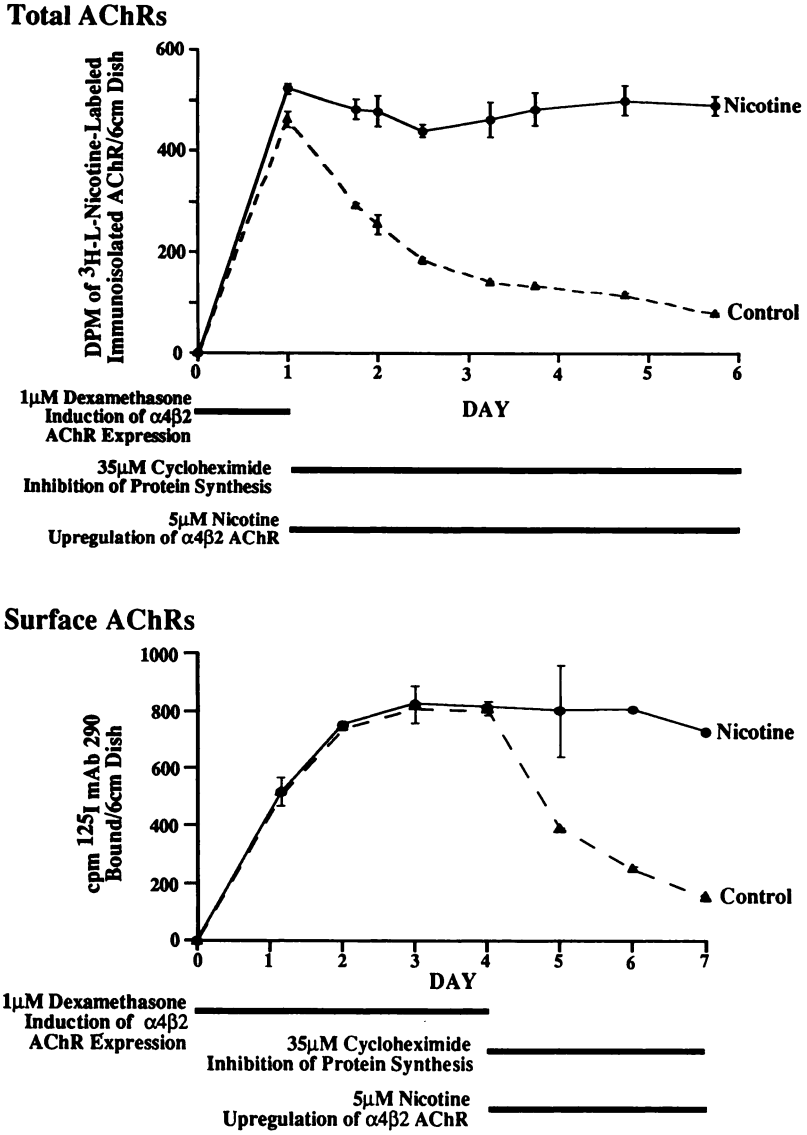

Fig. 12. Decrease by nicotine of the turnover rate of $\alpha4\beta2$ AChRs in M10 cells. Cycloheximide was used to inhibit the synthesis of new $\alpha4\beta2$ AChRs in dexamethasone-induced M10 cells. This concentration of cycloheximide, if added at the time of dexamethasone induction, prevented all detectable synthesis of new $\alpha4\beta2$ AChRs after 3 days. *Top*, cycloheximide was added 1 day after dexamethasone induction. The total amount of $\alpha4\beta2$ AChRs remaining in cells with or without nicotine treatment was then measured on subsequent days by L-[^{3}H]nicotine binding to immunoisolated solubilized $\alpha4\beta2$ AChRs. *Bottom*, dexamethasone induction of $\alpha4\beta2$ AChR synthesis was allowed to maximize over 4 days before nicotine was added to one set of cultures. In this case only surface AChRs were measured by binding of an ^{125}I-mAb to $\beta2$ subunits in intact cells.

the noncompetitive antagonist chlorisondamine causes blockage of AChR function, which persists for many days, but does not prevent nicotine-induced up-regulation of [^{3}H]nicotine binding sites (29). The failure of chlorisondamine to cause up-regulation by itself argues that not all noncompetitive inhibitors act by producing the conformation change induced by mecamylamine that triggers up-regulation. In brain, nicotine present chronically may act as a time-averaged antagonist, leaving a mixture of activatable, reversibly desensitized, and irreversibly desensitized $\alpha4\beta2$ AChRs, resulting in tolerance due to net decreased functional AChRs despite an increase in AChR protein. Chronic nicotine presumably produces its addictive effects through periodic stimulation of the activatable and reversibly desensitized AChRs.

Pharmacological properties required for up-regulation do not correlate precisely with those of either the activatable or detergent-solubilized desensitized state. Examination of Fig. 6 and Table 1 reveals that the concentrations at which agonists like nicotine induce up-regulation are much closer to the micromolar range in which they activate $\alpha4\beta2$ AChRs than the nanomolar range in which they bind to these AChRs at equilibrium after detergent solubilization and immunoisolation. However, the rank order of efficacy for up-regulation (cytisine ≈ nicotine > DMPP ≫ carbamylcholine) is the same as the rank order for equilibrium binding to desensitized $\alpha4\beta2$ AChRs but different, in the case of DMPP, from the rank order for activation of $\alpha4\beta2$ AChRs (cytisine > DMPP > nicotine ≫ carbamylcholine).

The rate of degradation of $\alpha4\beta2$ AChRs in M10 cells is decreased by exposure to nicotine (Fig. 12). This was demonstrated by first blocking the synthesis of new $\alpha4\beta2$ AChRs with the protein synthesis inhibitor cycloheximide and then measuring the amount of $\alpha4\beta2$ AChR remaining at subsequent times. The concentration of cycloheximide used, when added at the time of dexamethasone induction, prevents any detectable synthesis of AChRs. When AChR synthesis is induced by dexamethasone and then cycloheximide is added, AChRs turn over more rapidly in control cells than in cells treated also with nicotine. The nicotine-induced reduction in turnover of the $\alpha4\beta2$ AChRs persists for several days. The AChRs that turn over slowly in the presence of nicotine remain on the cell surface, where they can be labeled by ^{125}I-mAb in intact cells. This effect on degradation rate is large enough to account for the sustained increase in $\alpha4\beta2$ AChRs observed in M10 cells

treated with nicotine or in the brains of animals treated with nicotine.

The precise mechanism by which ligand-induced changes in $\alpha4\beta2$ AChRs reduce their susceptibility to turnover remains to be determined. One possible mechanism would be a conformation change that affects the large cytoplasmic domain of the $\alpha4\beta2$ AChR, altering how it interacts with endocytic and proteolytic mechanisms involved in turnover.

Up-regulation by agonists is not a universal property of all AChR subtypes. Muscle $\alpha1\beta1\gamma\delta$ AChRs have been reported to be down-regulated ~40% by chronic exposure to nicotinic agonists in culture (31–33). However, $\alpha1\beta1\gamma\delta$ AChRs expressed in the TE671 cell line are up-regulated by nicotine (34, 35). The $\alpha3\beta4\alpha5$ AChRs (36) expressed in chick ciliary ganglion neurons are reduced ~30% by chronic exposure of cultures to carbamylcholine (37). Brain α-bungarotoxin binding sites (probably $\alpha7$ AChRs) (38) are up-regulated by nicotine treatment (19), but to a lesser degree than are $\alpha4\beta2$ AChRs. Studies of these various AChR subtypes expressed in transfected fibroblasts or *Xenopus* oocytes, as we have performed here, could permit determination of the extent to which these regulatory effects are intrinsic properties of the various AChR subtypes or to what extent they reflect properties of the cells in which the AChRs are normally expressed.

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