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Differential Desensitization Properties of Rat Neuronal Nicotinic Acetylcholine Receptor Subunit Combinations Expressed in *Xenopus laevis* Oocytes

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KEY WORDS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; EGTA, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); OR2, oocyte Ringer's solution; MOR2, modified oocyte Ringer's solution.

SUMMARY

1. Chronic administration of nicotine up-regulates mammalian neuronal nicotinic acetylcholine receptors (nAChRs). A key hypothesis that explains up-regulation assumes that nicotine induces desensitization of receptor function. This is correlated with behaviorally expressed tolerance to the drug.

2. The present experiments were conducted to: (a) obtain information on the nicotine-induced desensitization of neuronal nAChR function, a less understood phenomenon as compared to that of the muscle and electric fish receptor counterparts; (b) test the hypothesis that different receptor subunit combinations exhibit distinct desensitization patterns.

3. *Xenopus laevis* oocytes were injected with mRNAs encoding rat receptor subunits $\alpha 2$, $\alpha 3$, or $\alpha 4$ in pairwise combination with the $\beta 2$ subunit. The responses to various concentrations of acetylcholine (ACh) or nicotine were analyzed by the two electrode voltage clamp technique.

4. Concentration-effect curves showed that nicotine was more potent than

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ACh for all the receptor subunit combinations tested. Only the $\alpha4\beta2$ combination exhibited a depression of the maximum effect at concentrations higher than 20 μM nicotine.

5. After a single nicotine pulse, receptor desensitization (calculated as a single exponential decay) was significantly slower for $\alpha4\beta2$ than for either $\alpha3\beta2$ or $\alpha2\beta2$.

6. Concentrations of nicotine that attained a near maximum effect were applied, washed, and re-applied in four minute cycles. The responses were calculated as percentages of the current evoked by the initial application. Following 16 minutes of this protocol, the $\alpha4\beta2$ combination showed a greater reduction of the original response as compared to the $\alpha2\beta2$ and $\alpha3\beta2$ subunit combinations. Taking points 5 and 6 together, these experiments suggest that the $\alpha4\beta2$ receptor subtype desensitizes at a slower rate and remains longer in the desensitized state.

7. Because $\alpha4\beta2$ is the main receptor subunit combination within the brain and is up-regulated by nicotine, our data may be important for understanding the molecular basis of tolerance to this drug.

INTRODUCTION

Chronic administration of the cholinergic agonist nicotine results in an increase of rodent neuronal nicotinic acetylcholine receptors (nAChRs) (Marks *et al.*, 1983; Schwartz and Kellar, 1985). Because receptor up-regulation occurs after chronic exposure to antagonists rather than to agonists, the increase in nAChRs can be explained by assuming that nicotine acts as a time-averaged functional antagonist promoting receptor desensitization (Marks *et al.*, 1983; Schwartz and Kellar, 1985).

The multiplicity of neuronal nAChRs (Patrick *et al.*, 1993; Sargent, 1993) and their distinct localization within the mammalian brain (Wada *et al.*, 1989) suggest that one or more receptor subtypes might exhibit particular desensitization characteristics when exposed to cholinergic agents. An investigation of this hypothesis may be relevant for understanding the molecular basis of nicotine tolerance (Ochoa *et al.*, 1990; Ochoa, 1994).

Cholinergic agonist-induced desensitization of neuronal nAChRs is still ill defined as compared to this process in muscle and electric organ nAChRs. While peripheral nAChRs consist of four subunits (i.e., α, β, γ, and δ), neuronal nAChRs are composed of only α and β (also called non-α) subunits. Major advances in the molecular cloning and functional expression of neuronal nAChRs have greatly facilitated the study of nicotine-induced desensitization of these receptors at the cellular and molecular level (Sargent, 1993; Patrick *et al.*, 1993).

To date, recombination DNA technology has defined seven types of rat α subunits ($\alpha2$-$\alpha7$ and $\alpha9$) and three types of rat β subunits ($\beta2$ to $\beta4$) that are expressed in different combinations throughout the CNS (Sargent, 1993; Patrick *et al.*, 1993; Elgoyhen *et al.*, 1994). In some cases, functional receptors can be assembled from one type of subunit (e.g., $\alpha7$), but it is likely that most neuronal

nAChRs consist of a pairwise combination of single type of α subunit with a single type of β subunit, and possibly with a third subunit (Corriveau and Berg, 1993). This complexity is increased by the fact that in some brain areas, several types of nAChR may coexist in a single neuron (Alkondon and Albuquerque, 1993).

Because most nAChRs are presynaptic (Wonnacott *et al.*, 1989; Wonnacott, 1991; Flores *et al.*, 1992; Wilkie *et al.*, 1993) which makes their *in situ* electrophysiological characterization very difficult, mRNAs corresponding to the different rat neuronal nAChR subunits were injected into *Xenopus laevis* oocytes (Leonard and Snutch, 1991). The activity of the expressed receptors upon exposure to nicotine and acetylcholine (ACh) was monitored by the two electrode voltage clamp technique and the rate of decay of the activated currents was determined.

Concentration-effect curves for ACh and nicotine from 50 nM to 1 mM were constructed for $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha4\beta2$. Previous studies from other laboratories did not explore nicotine concentration effects for $\alpha4\beta2$ above 5 μM.

Moreover, these receptor subunit combinations were chosen in these experiments for the following reasons: a) $\alpha4\beta2$ is the predominant form in the mammalian brain and is specifically up-regulated after chronic nicotine administration (Flores *et al.*, 1992); b) $\alpha2$, $\alpha3$, and $\alpha4$ form functional receptors when expressed in conjunction with β subunits (Boulter *et al.*, 1987; Ballivet *et al.*, 1988; Wada *et al.*, 1988; Duvoisin *et al.*, 1989; Bertrand *et al.*, 1990; Couturier *et al.*, 1990a; Couturier *et al.*, 1990b; Bertrand *et al.*, 1992; Vibat *et al.*, 1994), c) all the α subunits show regionalization within the rat brain, but $\beta2$ is widely distributed (Wada *et al.*, 1989) and may be a common structural element in neuronal nAChRs (Papke *et al.*, 1989; Hill *et al.*, 1993).

Our experiments show that, when compared to the $\alpha2\beta2$ or $\alpha3\beta2$ receptor subunit combinations, the $\alpha4\beta2$ subtype exhibited a significant depression of the maximum response at concentrations higher than 20 μM nicotine, a slower rate of nicotine-induced desensitization, and a more profound inactivation of the response after repetitive applications of the drug. Results of this research have been partially presented to the 24th meeting of the Society for Neuroscience (Vibat *et al.*, 1994).

MATERIALS AND METHODS

Materials

Restriction enzymes for linearization of plasmids containing cDNA were purchased from Promega (Madison, WI), New England Biolabs (Beverly, MA), and Pharmacia (Piscataway, NJ). The $m^7G(5')ppp(5')G$ used for capping mRNA transcripts were obtained from Pharmacia. The MEGAscript™ kit for *in vitro* transcription reactions was purchased from Ambion (Austin, TX). Media and antibiotic/antimycotic reagents used to maintain injected oocytes were obtained

from Gibco (Gaithersburg, MD). (-)-Nicotine (hydrogen tartrate salt), acetylcholine chloride, and collagenase were from Sigma (St. Louis, MO). Throughout this paper, nicotine stands for (-)-nicotine (hydrogen tartrate salt). All other reagents were purchased from standard suppliers.

Methods

In vitro Synthesis of mRNA Transcripts and Expression in Xenopus laevis Oocytes. The plasmids HYP16(9), containing the full length cDNA of the rat neuronal $\alpha 2$ subunit, PCA48E(3) containing rat neuronal $\alpha 3$, HYA23-1E(1) containing rat $\alpha 4$-1, and PCX49(1) with the rat neuronal $\beta 2$ subunit cDNA were generous gifts from Dr. Jim Boulter, the Molecular Neurobiology Lab, Salk Institute, La Jolla, CA. The mRNA transcripts were synthesized *in vitro* using linearized cDNA templates in the presence of SP6 or T7 RNA polymerase, rNTPs, and $m^7G(5')ppp(5')G$. The RNA transcripts were purified using Select-D(RF) columns (5 Prime-3 Prime Inc., Boulder, CO). Rat neuronal AChR $\alpha 2$, $\alpha 3$, or $\alpha 4$ mRNA was combined with $\beta 2$ mRNA in the ratio of 2:3 to a final concentration of 0.2 to 0.4 $\mu g/\mu l$.

Ovarian lobes were removed from anesthetized female *Xenopus laevis* via 1 cm long ventral incision, and the wounds were stitched with sterile cat gut. The occytes were defolliculated by incubation for 1.5 hours at room temperature with slow agitation in Ca^{+2}-free OR2 buffer (Wallace *et al.*, 1973) containing (in mM) 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 Na_2HPO_4, and 5 HEPES, pH 7.6, in the presence of 2 mg/ml collagenase (type IA). This was followed by manual removal of the follicle cell layers. Stage V and VI oocytes were chosen for mRNA injection. 50 nl of the RNA mixtures were injected into stage V or VI oocytes. Injected oocytes were maintained at 18–19°C in a medium of 50% Leibovitz's L-15 media, 0.4 mg/ml bovine serum albumin, plus 100U penicillin G (sodium salt)/ml, 100 mg streptomycin sulfate/ml, and 250 ng amphoterin B/ml. The medium was changed daily.

Electrophysiological Procedures. Three to six days after the injection of mRNA, voltage clamp experiments were carried out on the oocyte at room temperature using an Axoclamp 2A (Axon Instruments, Burlingame, CA) in the two electrode voltage clamp configuration. Microelectrodes were prepared from 1.5 mm OD/0.84 mm ID glass capillaries and filled with 3 M KCl. Voltage electrodes were 2 MΩ to 15 MΩ and current electrodes were 0.3 MΩ to 1.0 MΩ in resistance. After impalement into the oocyte, electrodes were compensated by the bridge balance circuit through series resistance. The oocytes were continuously perfused at a rate of 15 ml/min with MOR2 buffer (in mM): 82 NaCl, 2.5 KCl, 1 Na_2HPO_4, 5 $MgCl_2$, 0.2 $CaCl_2$, 5 HEPES, pH 7.4) supplemented with 2 μM atropine. Prior to voltage clamp experiments, agonist solutions were freshly prepared in Ca^{+2}-free MOR2 containing 0.5 mM EGTA and 2 μM atropine.

Data for concentration-effect curves were collected and analyzed by the SCAN and INPLOT programs (GraphPad Software, San Diego), respectively. The oocytes were held at a membrane potential of −80 mV. Curves were fit to

the equation $Y = 100/(1 + EC_{50}/A)^n$. Three to six oocytes were averaged to obtain each of the calculated plots.

First order rate constants (τ) for nicotine-induced desensitization were determined by subjecting oocytes which had not been previously exposed to the drug, to a single application of nicotine. This was performed at a holding potential of −80 mV. In order to normalize between the different receptor subunit combinations, concentrations that evoked 75% of the maximum current for each subtype were chosen. It is reasonable to assume that desensitization occurs at these concentrations. The traces were fit to a one exponential decay using SCAN and statistical analysis of the data was performed using INPLOT. For each receptor subunit combination, four to seven oocytes were used to determine the average τ and SEM. Statistically significant differences were calculated using InStat (GraphPad).

In measuring current-voltage (I/V) relationships of $\alpha4\beta2$, the oocyte membrane potential was initially held at −80 mV. CLAMPEX (Axon Instruments, Inc.) was then used to step the potential in a cycle from +100 mV for 100 ms to the desired voltage for 200 ms until a range from −120 mV to +100 mV was covered in 20 mV increments. Baseline responses obtained in the absence of agonist were subtracted from currents evoked by 0.3 μM or 40 μM nicotine. The same oocyte was used to generate I/V plots at both concentrations, and three oocytes were averaged.

The effect of multiple exposures of nicotine upon receptor inactivation were performed for each receptor subtype using submaximal concentrations of nicotine. The evoked current from the initial application was taken as 100%. Between drug applications, the oocyte was washed for four minutes in MOR2, pH 7.4, plus 2 μM atropine. Currents produced in subsequent cycles were calculated as percentages of the initial current and averaged for three to six oocytes per receptor subunit combination.

RESULTS

Different Neuronal nAChR Subunit Combinations Have Different Sensitivities to ACh and Nicotine

The neuronal nAChR subunit combinations expressed in *X. laevis* oocytes had different sensitivities to ACh and nicotine, as depicted in Figs. 1A–C and in Table I. The EC_{50} values, calculated from the concentration-effect curves (Table I) indicated that nicotine was a more potent agonist than ACh for all the receptor combinations tested.

An important finding was that the $\alpha4\beta2$ subunit combination showed an extreme depression of the maximum effect upon nicotine application at concentrations greater than 20 μM (Fig. 1C). This nicotine-induced effect was specific for $\alpha4\beta2$ as compared to the $\alpha2\beta2$ and $\alpha3\beta2$ subunit combinations (cf. Fig. 1C and Fig. 1A–B). In addition, ACh did not produce a depression of the maximum in any of the three receptor subunit combinations (Fig. 1A–C).

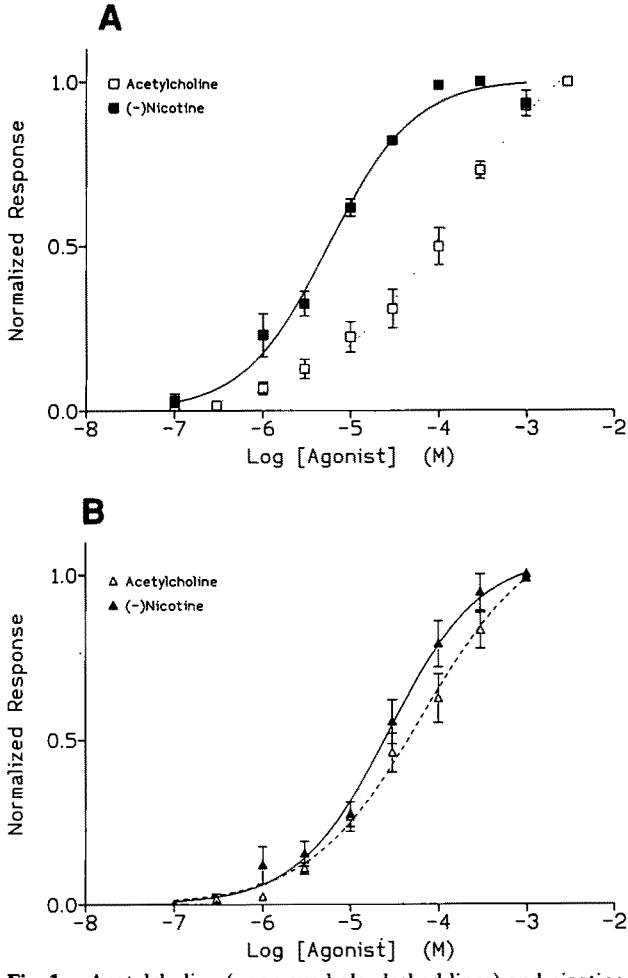

Fig. 1. Acetylcholine (open symbols, dashed lines) and nicotine (filled symbols, solid lines) concentration-effect curves for various neuronal nAChR subunit combinations. (A) $\alpha 2\beta 2$, (B) $\alpha 3\beta 2$, (C) $\alpha 4\beta 2$. Each curve represents the average of three to six oocytes. The response was normalized to the maximum current elicited in each oocyte by the corresponding cholinergic agonist.

This depression of the maximum effect in $\alpha 4\beta 2$ could be due to receptor desensitization and/or channel blockade. In ligand-gated ion channels, drug-effected blockade exhibits voltage dependence (Carter and Oswald, 1993). If voltage-dependent blockade exists at high concentrations of nicotine, I/V plots obtained at high concentrations of nicotine would most likely be different from plots at low concentration.

In order to test this hypothesis, the experiments depicted in Fig. 2 were conducted at 0.3 μM and at 40 μM (Fig. 1C). First, the rectification properties of $\alpha 4\beta 2$ at both concentrations were similar to those described by Charnet to ACh

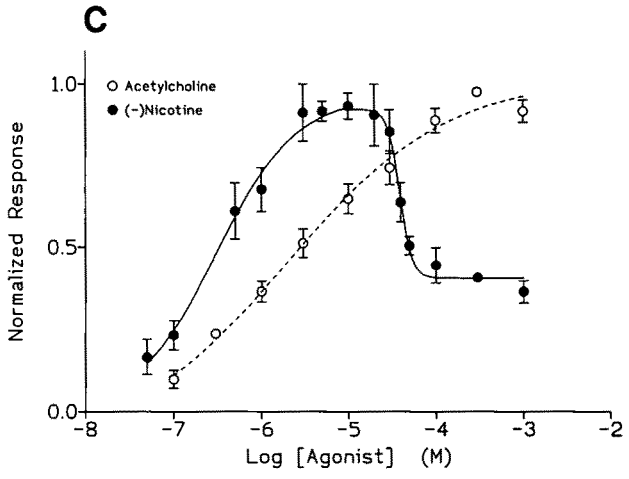

Fig. 1. (*Continued.*)

(Charnet *et al.*, 1992). In addition, when the responses were normalized to the maximum currents observed at each respective concentration, the I/V curves were identical (Fig. 2).

Different Neuronal nAChR Subunit Combinations Have Different Desensitization Characteristics

The effect produced by a single application of either ACh or nicotine was measured for $\alpha2\beta2$, $\alpha3\beta2$, and $\alpha4\beta2$ and its rate of decay was fitted to a single exponential (see Materials and Methods). Figures 3A–C show two electrode voltage clamp traces of typical responses to concentrations of nicotine that induced 75% of the maximal response as determined from Fig. 1A–C. Again, $\alpha4\beta2$ revealed a striking difference when compared to $\alpha2\beta2$ and $\alpha3\beta2$: while nicotine was still present in the perfusing buffer, the rate of decay of the nicotine-induced current for $\alpha4\beta2$ was $56\,sec^{-1}$, whereas the rates of decay for the currents from $\alpha2\beta2$ and $\alpha3\beta2$ were significantly faster ($15\,sec^{-1}$ and $17\,sec^{-1}$ respectively; Table II). The maximum amplitude of the current induced by ACh

Table 1. The Nicotine and Acetylcholine EC_{50} Values for Various Neuronal nAChR Combinations

Subunit combination	Nicotine (μM)	Acetylcholine (μM)
$\alpha2\beta2$	6[a]	152
$\alpha3\beta2$	28	65
$\alpha4\beta2$	0.3	3

[a] Mean EC_{50}s were calculated from the concentration-effect curves depicted in Fig. 1A–C. For experimental details see text of Fig. 1A–C.

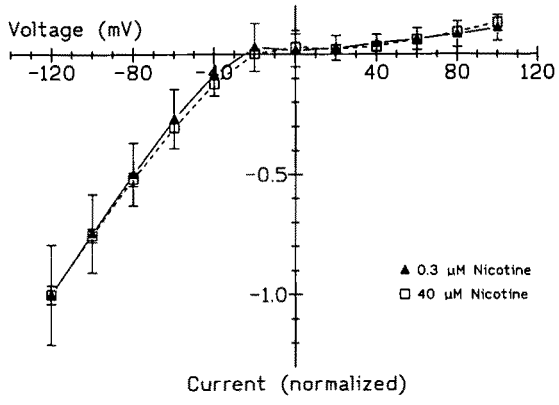

Fig. 2. I/V curves of $\alpha 4\beta 2$ at two concentrations of nicotine: 0.3 μM (filled triangles, solid line) and 40 μM (open squares, dashed lines). Currents induced by the application of nicotine at these concentrations were determined at membrane potentials ranging from −120 mV to +100 mV. The concentrations used to construct these curves were the EC_{50} (0.3 μM) and a concentration at which significant depression of the maximum current was observed (40 μM) as determined from Fig. 1C. The maximum currents seen at −120 mV for 0.3 μM nicotine and 40 μN nicotine were −601 nA and −1856 nA, respectively. Each plot was normalized to the maximum current observed at that concentration.

was, in all cases, of greater magnitude than that evoked by nicotine (data not shown).

Progressive Desensitization of the Nicotine-Induced Effect is Observed upon the Repetitive Application of the Drug

A protocol of repetitive applications of nicotine (see Materials and Methods) was designed to further explore differences in desensitization behavior between $\alpha 4\beta 2$ and the other two receptor subtypes. Submaximal nicotine concentrations (Fig. 1A–C) were applied, washed, and re-applied in four minute cycles (Fig. 4).

After the initial response, each data point for the $\alpha 4\beta 2$ combination was significantly lower than either of the other two subtypes ($p < 0.05$). With respect to $\alpha 2\beta 2$ and $\alpha 3\beta 2$, the data were not significantly different at any time point except at 16 minutes (Fig. 4).

DISCUSSION

The subunit composition of native neuronal nAChRs, their topology within the neuron, and their distribution throughout different regions of the brain may define specific targets involved in dependence to nicotine (Ochoa, 1994). Also,

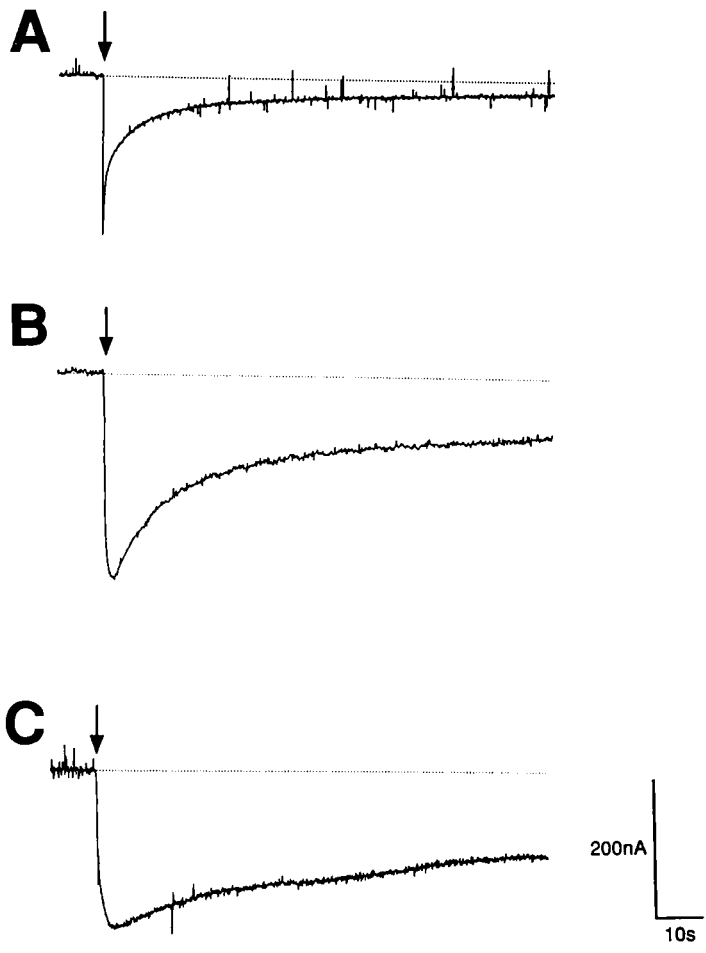

Fig. 3. Typical traces of nicotine induced currents for various neuronal nAChR subunit combinations. Currents were measured at nicotine concentrations determined to produce 75% maximal response for that particular subunit combination (see Fig. 1A–C). (A) $\alpha 3\beta 2$ at 60 μM nicotine, (B) $\alpha 2\beta 2$ at 20 μM nicotine, (C) $\alpha 4\beta 2$ at 2 μM nicotine. Nicotine was applied at the arrow and was not removed from the bath. First order rate constants for current decay (τ) were fitted to one exponential (for details see Materials and Methods).

the diversity of receptor subtypes may influence modes by which neuronal nAChR are regulated by external agents (Changeux, 1990).

The rate of desensitization of various types of nAChRs is influenced by their subunit composition (Couturier *et al.*, 1990a; Luetje and Patrick, 1991; Cachelin and Jaggi, 1991; Gross *et al.*, 1991; Morales and Sumikawa, 1992). Thus, it is reasonable to hypothesize that different neuronal nAChR subunit combinations have distinct desensitization patterns in the presence of nicotine (Ochoa *et al.*, 1990).

We focused on three neuronal nAChR α subunits ($\alpha 2$–$\alpha 4$) and one

Table II. Nicotine-Induced Desensitization: Decay Time Constants (τ) For Various Neuronal nAChR Combinations[a]

Subunit combination	τ (sec^{-1})
$\alpha2\beta2$	15 ± 3 ($n = 5$)*
$\alpha3\beta2$	17 ± 4 ($n = 4$)**
$\alpha4\beta2$	56 ± 21 ($n = 12$)

[a] The time constant τ was obtained at nicotine concentrations calculated to induce currents at 75% of the maximal response. The concentrations used for the various subunit combinations were as follows: (1) $\alpha2\beta2$: 20 μM, (2) $\alpha3\beta2$: 60 μM, and (3) $\alpha4\beta2$: 2 μM. For the $\alpha2\beta2$ and $\alpha3\beta2$ combinations, τ values were not statistically different. P values were calculated from non-parametric one-way ANOVA followed by the Dunn's post test.

* Significantly lower than τ for $\alpha4\beta2$ ($p < 0.01$).

**Significantly lower than τ for $\alpha4\beta2$.

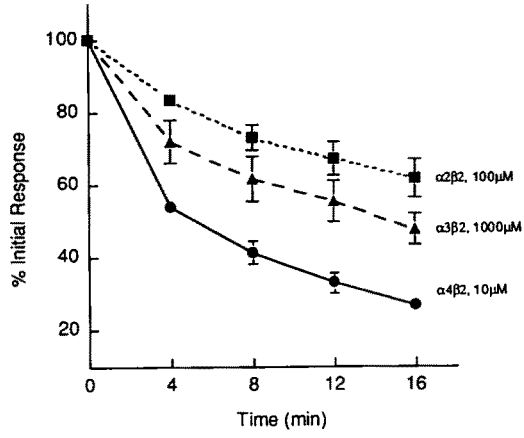

Fig. 4. Inactivation of nicotine-induced currents after repeated applications of the drug. Submaximal nicotine concentrations for each combination (indicated for each curve) were applied to the oocytes for 20 sec and the recorded initial current was taken as 100%. The oocytes were washed for 4 min and another 20 sec application of the same nicotine concentration and a wash followed. The second response was calculated as a percentage of the initial response. The whole application-washing procedure was repeated three more times. Data points represent the mean ± SEM of three to six oocytes for each receptor combination. $\alpha2\beta2$ (dotted line and filled squares); $\alpha3\beta2$ (dashed line and filled triangles); $\alpha4\beta2$ (solid lines and filled circles). Each experimental data point at each time for the $\alpha4\beta2$ combination was significantly lower than each data point for any of the other two combinations at the same times ($p < 0.05$). For the $\alpha2\beta2$ and the $\alpha3\beta2$ combinations, data points were not significantly different except for the last data point pair ($p < 0.05$). P values were calculated by one-way ANOVA followed by the Fischer's protected LSD test.

neuronal nAChR β subunit (β2) based on the following findings. The α2, α3, and α4 subunits form functional receptors with β2 and β4 subunits (Boulter *et al.*, 1987; Ballivet *et al.*, 1988; Wada *et al.*, 1988; Duvoisin *et al.*, 1989; Bertrand *et al.*, 1990; Couturier *et al.*, 1990a; Couturier *et al.*, 1990b; Bertrand *et al.*, 1992; Vibat *et al.*, 1994). In addition, the rat mRNAs for the α5 and α6 subunits do not express functional receptors when co-injected with other α and β mRNAs into *Xenopus* oocytes (Boulter *et al.*, 1990). Also, the α7 subunit forms a functional homomeric receptor that presumably does not combine with β subunits (Couturier *et al.*, 1990a; Seguela *et al.*, 1993). While all the α subunits show distinct (although overlapping) regionalization within the rat brain, the β2 subunit is widely distributed (Wada *et al.*, 1989) and may be a common structural element in neuronal nAChRs (Papke *et al.*, 1989; Hill *et al.*, 1993).

Immunoprecipitation of rat brain high affinity nicotine binding sites using polyclonal antisera in conjunction with tritiated cytisine has shown that the predominant form of the neuronal nAChR consists of α4 and β2 subunits (Flores *et al.*, 1992). This subunit combination is specifically up-regulated after chronic nicotine administration (Flores *et al.*, 1992), making it a plausible target of nicotine effects within the brain.

Presynaptic localization for most neuronal nAChRs is well documented (Wonnacott *et al.*, 1989; Wonnacott, 1991; Flores *et al.*, 1992; Wilkie *et al.*, 1993). For example, nicotine exposure coincides with desensitization of nicotine-evoked, nAChR-dependent transmitter release from whole organs (Schiavone and Kirpekar, 1982), tissue slices (Yu and Wecker, 1994), cultured cells (Higgins and Berg, 1988), and synaptosomal preparations (Tandon and Ochoa, 1992; Ochoa and O'Shea, 1994; Marks *et al.*, 1994; Grady *et al.*, 1994; Rowell and Hillebrand, 1994).

An electrophysiological study of the desensitization characteristics of native neuronal nAChRs is extremely difficult due to the aforementioned preferential presynaptic localization of these receptors. Therefore, *Xenopus laevis* oocytes were chosen for transiently expressing our neuronal nAChR subunit combinations. It is widely assumed that these combinations are similar in structure and function to those found in neuronal membranes.

As mentioned before, the rat (or chick) α2, α3, and α4 subunits can each be co-expressed with the rat (or chick) β2 and β4 subunits in *X. laevis* oocytes to yield functional receptor channels which respond to either acetylcholine or nicotine (Boulter *et al.*, 1987; Ballivet *et al.*, 1988; Wada *et al.*, 1988; Duvoisin *et al.*, 1989; Bertrand *et al.*, 1990; Couturier *et al.*, 1990a; Couturier *et al.*, 1990b; Bertrand *et al.*, 1992; Vibat *et al.*, 1994). Likewise, the expressed receptor subunit combinations showed depolarizing responses when stimulated with nicotine and ACh (Fig 1. A–C). In addition, α4β2 exhibited rectification (Fig. 2) as expected for neuronal nAChRs (Charnet *et al.*, 1992; Sands and Barish, 1992). Concentration-effect curves revealed that nicotine was more potent than ACh for activating all of the expressed receptor subtypes studied (Fig. 1A–C, Table I).

In addition, the α4β2 combination (but not α2β2 or α3β2, Fig. 1A,B) exhibited a depression of the maximum effect at high concentrations of nicotine (Fig. 1C). Such a bell-shaped form is observed in concentration-effect curves that

measure nicotine and cholinergic agonist-activated ion flux in cultured cells (Robinson and McGee, 1985; Boyd, 1987; Lukas, 1993).

In contrast to these observations in rat receptor subunits, chicken $\alpha 3$ non-$\alpha 1$ (analogous to rat $\alpha 3\beta 2$), but not chicken $\alpha 4$ non-$\alpha 1$ (analogous to rat $\alpha 4\beta 2$) exhibits bell-shaped curves (Gross *et al.*, 1991). It is unclear whether these dissimilarities between analogous subunit combinations can be due to species differences.

The results from Fig. 1A–C may indicate that nicotine-induced desensitization predominates over nicotine-induced activation at high concentrations of the drug in $\alpha 4\beta 2$ but not in $\alpha 2\beta 2$ or $\alpha 3\beta 2$. Alternatively, this depression of the maximum may be caused by an agonist-effected blockade of the nAChR channel at high drug concentrations. Ligand-gated ion channels show the phenomenon of voltage dependent, drug-effected blockade (Nelson and Albuquerque, 1994).

Consequently, I/V protocols were performed on $\alpha 4\beta 2$ (Fig. 2) using equally effective concentrations of nicotine as determined from the concentration-effect curves (i.e. 0.3 μM and 40 μM, see Fig. 1C). When the responses were normalized to the maximum currents observed at each respective concentration, the I/V curves were identical (Fig. 2). Although this result may be interpreted as nicotine-induced desensitization (but not voltage-dependent blockade) at 40 μM drug, a more detailed electrophysiological analysis at the single channel level is required for the following reasons.

Patch clamp analaysis has revealed that $\alpha 4\beta 2$ has more than a single conductance state (34 pS, 22 pS, and 12 pS) at 1.0 μM ACh, and 13°C, (Charnet *et al.*, 1992). To date, no single channel data for ACh at concentrations higher than 4 μM have been reported, probably due to the fast desensitization exhibited by the neuronal receptor subunit combinations expressed in *Xenopus* oocytes. Experiments using the outside-out configuration with fast perfusion of high concentrations of ACh and nicotine (Naranjo and Brehm, 1993) are underway.

To further explore differences in the desensitization properties of $\alpha 4\beta 2$ as compared to $\alpha 2\beta 2$ and $\alpha 3\beta 2$ experiments were performed at submaximal concentrations of nicotine (Figs. 3 and 4; Table II). The $\alpha 3\beta 2$ and the $\alpha 2\beta 2$ combinations desensitize faster after a single and constant application of nicotine as compared to the $\alpha 4\beta 2$ subtype (Fig. 3 and Table II). This is consistent with faster desensitization rates observed in chicken $\alpha 3$-non $\alpha 1$ as compared with chicken $\alpha 4$-non $\alpha 1$ (Gross *et al.*, 1991). Furthermore, repetitive applications of nicotine (Fig. 4), showed that the response to nicotine for $\alpha 4\beta 2$ was significantly diminished as compared to $\alpha 2\beta 2$ and $\alpha 3\beta 2$ (Fig. 4).

Taken altogether, the data from Fig. 3, Table II and Fig. 4 suggest that the $\alpha 4\beta 2$ receptor subunit combination is desensitized by nicotine at a slower rate that their $\alpha 2\beta 2$ and $\alpha 3\beta 2$ counterparts, but it also remains longer in its desensitized form when successively challenged with the drug. This may have relevance to the chronic administration of nicotine seen in smokers (Ochoa, 1994).

Two other possible explanations for the decreased responses depicted in Fig. 4 were considered: rundown (the progressive decline of the preparation over the course of an experiment) and agonist-effected channel block. Because all the

oocytes used in the repetitive application protocols (Fig. 4) were from the same batch, it is reasonable to assume that a similar degree of rundown occurred throughout all the experiments. Therefore, it is unlikely that the observed difference between $\alpha4\beta2$ compared to $\alpha2\beta2$ and $\alpha3\beta2$ is exclusively determined by this phenomenon. It is noteworthy that rundown occurs independently of the application of agonist (Lester and Dani, 1994).

In conclusion, we have demonstrated that the $\alpha4\beta2$ neuronal nAChR subunit combination is preferentially activated by nicotine over ACh, making $\alpha4\beta2$ a preferential target of nicotine effects within the brain. The functional $\alpha4\beta2$ receptor subtype exhibits different desensitization characteristics as compared to the $\alpha2\beta2$ and $\alpha3\beta2$ combinations: a depression of the maximum in concentration-effect curves at high concentrations of nicotine, a slow desensitization after a single application of the drug, and a greater relative depression of the elicited effect after consecutive applications of nicotine.

Our findings are relevant to understanding the molecular basis of tolerance to nicotine seen after protracted administration of the drug (Marks *et al.*, 1983; Schwartz and Kellar, 1985; Ochoa, 1994).

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