1999

Desensitization and upregulation of neuronal nicotinic acetylcholine receptors.

Catherine Pichon Fenster
University of Alabama at Birmingham

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DESENSITIZATION AND UPREGULATION OF NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS

by

CATHERINE PICHON FENSTER

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama a Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

1999

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D. Program Neurobiology

Name of Candidate Catherine Pichon Fenster

Committee Chair Michael W. Quick

Title Desensitization and Upregulation of Neuronal Nicotinic Acetylcholine Receptors

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Prolonged exposure to nicotine obtained from tobacco is associated with an upregulation in the number of some brain nicotinic acetylcholine receptors (nAChRs) and a concomitant downregulation of receptor function. Functional downregulation, and possibly even numerical upregulation, is thought to be the consequence of agonist-induced desensitized states. Because desensitization and upregulation of some nAChR subtypes occurs with chronic exposure to nicotine at levels obtained from tobacco, these processes are likely to contribute to nicotine's addictive properties. In addition, nAChR desensitization may be regulated by physiologically relevant factors such as extracellular Ca^{2+} and intracellular second messengers. Therefore, the current studies were undertaken to investigate the following hypotheses: (1) nAChR desensitization is influenced by subunit composition; (2) desensitization and upregulation in the number of surface nAChRs are related phenomena; and (3) nAChRs desensitization is influenced by extra-cellular Ca^{2+} and protein kinase-C (PKC).

These hypotheses were examined for rat nAChRs expressed in *Xenopus* oocytes. Functional desensitization was assessed using whole-cell, two-electrode voltage-clamp techniques. Upregulation of surface receptor number was estimated from a [^{3}H]nicotine binding assay performed on intact oocytes. Consistent with the first hypothesis, we find that the $\alpha4$ nAChR subunit contributes a high affinity for nicotine induced desensitization (relative to $\alpha3$ and $\alpha7$ nAChR subunits) and that the $\beta2$ subunit contributes, in part, to faster desensitization kinetics. As predicted if a desensitized conformation initiates upregulation of nAChR number (hypothesis 2), we find that, for

surface α4β2 nAChRs, nicotine dose-dependencies for upregulation largely coincide with that of both functional desensitization and equilibrium [^{3}H]nicotine binding to nAChRs on intact cells. Upregulation and functional desensitization also exhibit similar nicotine dose-dependencies for α3β4 nAChRs, a receptor subtype with lower apparent affinity for functional desensitization than that of α4β2 nAChRs. These results imply that desensitization may serve as a common trigger for upregulation of different nAChR subtypes. In addition, our results suggest that the "desensitization hypothesis" may apply only to upregulation of surface nAChRs because upregulation of intracellular α4β2 nAChRs requires much higher concentrations of nicotine than are required for either desensitization of surface receptors or [^{3}H]nicotine equilibrium binding to membrane homogenates. Consistent with the third hypothesis, recovery from desensitization was accelerated for α4β2 nAChRs in the presence of extracellular Ca^{2-}. Recovery from desensitization was also strongly influenced by the balance of PKC and phosphatase activity. For example, recovery from desensitization was enhanced in the presence of activators of PKC or phosphatase inhibitors and was reduced in the presence of inhibitors of PKC. Phosphorylation of a PKC consensus sequence site on the α4 subunit (serine 336) is likely to be involved because desensitization recovery rates were reduced for mutant α4β2 nAChRs lacking this site.

DEDICATION

This work is dedicated to my husband and my "soul-mate," Steven Daniel Fenster.

ACKNOWLEDGMENTS

First, I would like to thank my mentor, Dr. Michael Quick, who has provided knowledge, wisdom, and (most importantly) encouragement. His patience and understanding have helped me to survive the past year. In addition, I would like to thank Dr. Robin A. J. Lester, who has guided my research and whose passion for science has been contagious. Both these men have and will serve as valuable role models. I have been fortuitous to work with and for two men that I honestly admire.

Next, I would like to express my gratitude toward the other members of my committee, Drs. David Weiss, Kent Keyser, and Edwin Blalock. Each of these members has contributed valuable suggestions and advice. I must also mention several rotation and work-study students who have participated in some of the experiments presented in this dissertation: Terri Whitworth, Julie Parker, Elise Sheffield, Felicia Rains, and Brent Noerager. Matthew Beckman, a fellow graduate student in the Quick lab, has also contributed by creating a mutant subunit protein. He and Eve Bernstein (fellow "Quick labers") have, in general, made lab work enjoyable. Finally, I would like to thank the faculty and staff of the Neurobiology Department who contributed to my education at UAB.

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LIST OF ABBREVIATIONS

Ba2+	Barium
Ca2+	Calcium
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
HEK	Human embryonic kidney
Na+	Sodium
nAChR	Nicotinic acetylcholine receptor
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
PCR	Polymerase chain reaction

INTRODUCTION

Addiction to nicotine in the form of tobacco use is a major risk factor for cancer, disease, and premature death (Peto and Doll, 1992). These known risks, combined with the prevalence of tobacco use, makes nicotine addiction a leading public health issue. Although nicotine is an addictive drug, it may have some important therapeutic applications. For example, nicotine appears to reduce cognitive deficits associated with Alzheimer's disease (Arneric et al., 1995). Nicotine is also being investigated for its ability to alleviate symptoms associated with several other disorders, including Parkinson's disease, schizophrenia, Tourette's syndrome, sleep apnea, ulcerative colitis, pain, and attention–deficit disorder (Levin et al., 1992; Freedman et al., 1994; Arneric et al., 1995; Shytle et al., 1996; Lindstrom, 1997).

Neuronal nAChRs

The addictive and therapeutic properties of nicotine are mediated through its interaction with neuronal-type nAChRs. Neuronal nAChRs are members of a genetic super-family of transmitter-gated ion channels that includes neurotransmitter receptors for γ-aminobutyric acid, glycine, serotonin, and acetylcholine (Betz, 1990). The neuromuscular-type nAChR is considered an archetype of this receptor family because it was the first to be extensively characterized, both structurally and functionally (see Role, 1992). An extensive characterization of neuronal nAChRs has proved more difficult because they are a functionally and structurally diverse receptor group with relatively diffuse patterns of distribution. However, an understanding of how neuronal nAChRs respond to nicotine exposure is essential to understanding the molecular basis of nicotine's behavioral effects (Balfour, 1994; Dani and Heinemann, 1996).

The Physiological Role of nAChRs in the Central Nervous System

To appreciate the effects of the interaction of nicotine with nAChRs, it is necessary to have a basic understanding of the physiological role of neuronal nAChRs. The role of brain nAChRs in normal brain physiology is just beginning to be understood (Colquhoun and Patrick, 1997). The importance of nAChRs in brain function is implied by the wide variety of behavioral and cognitive effects of nAChR agonists and antagonists. For example, nicotine and other nAChR agonists have been shown to enhance attention, reduce anxiety, improve some forms of memory and learning, and may even act as a mild analgesic (McGehee and Role, 1996; Lindstrom, 1997; Marubio et al., 1999; Zoli et al., 1999). Recent studies of mutant mice that lack specific nAChR subunits have implicated individual subunits as components of nAChRs involved in several brain functions including pain perception, short-term memory, learning, and nicotine addiction (Picciotto et al., 1998; Marubio et al., 1999; Xu et al., 1999; Zoli et al., 1999).

In the central nervous system (CNS), it has been difficult to demonstrate the involvement of postsynaptic nAChRs in classical fast excitatory transmission. However, a few recent studies have successfully demonstrated the existence of somatodendritic nAChRs that, when activated, generate synaptic currents (Zhang et al., 1999). Many brain nAChRs are instead found at perisynaptic and presynaptic cellular locations where they function to modulate neurotransmitter release (McGehee and Role, 1996; Role and Berg, 1996; Wonnacott, 1997). Activation of perisynaptic nAChRs facilitates neurotransmitter release by activating local voltage-gated Na^+ and Ca^{2+} channels (Rathouz and Berg, 1994). Ca^{2+} influx through activated nAChRs at the presynaptic nerve terminal may directly trigger and/or facilitate neurotransmitter vesicle release. Activation of nAChRs by exogenous agonist application enhances the release of several neurotransmitters including norepinephrine, dopamine, γ-aminobutyric acid, serotonin, acetylcholine, and glutamate (Clark, 1993; Gray et al., 1996; Pontieri et al., 1996; Role and Berg, 1996; Lena and Changeux, 1997).

The relevance of nAChR modulation of neurotransmitter release during normal neurotransmission has not been established but may be inferred from the abundance of cholinergic projections in brain. The physiological and behavioral effects of nicotine, however, have been attributed to its ability to enhance neurotransmitter release by activating nAChRs. For example, glutamate release in the hippocampus (a brain center associated with learning and memory) is enhanced by nicotine (Gray et al., 1996), an action that may underlie the ability of nicotine to stimulate awareness and memory. Additionally, nicotine's addictive properties have been attributed to its activation of nAChRs on dopaminergic neurons in the mesolimbic "central reward pathway." Activation of these nAChRs enhances dopamine release in the nucleus accumbens (Dani and Heinemann, 1996; Pontieri et al., 1996), a brain region in which dopamine transmission has been linked to the reinforcing actions of many drugs of abuse. It is also important to note that, because nAChRs are highly permeable to Ca^{2+} (Mulle et al., 1992a; Vernino et al., 1992; Seguela et al., 1993; Rathouz and Berg, 1994), nAChR activation results in transient elevations in intracellular Ca^{2+} levels (Vernino et al., 1992; Khiroug et al., 1997; Zhang et al., 1999). Therefore, in addition to facilitating neurotransmitter release, nAChR activation can influence the activity of Ca^{2+}-dependent ion channels and/or second messenger pathways (Vijayaraghavan et al., 1990; Khiroug et al., 1998). Through activation of these pathways, which modulate protein function and even gene expression, nAChR activation may have long-term cellular consequences (Chan and Quik, 1993; Amador and Dani, 1995; Role and Berg, 1996).

Nicotine Addiction

With tobacco use, arterial nicotine levels are estimated to peak around 1 μM (Benowitz et al., 1989). This initial rise in nicotine activates nAChRs that stimulate dopamine release in the mesolimbic dopamine pathway to provide a rewarding/reinforcing effect (Picciotto et al., 1998). While nAChR activation has been

linked to the behavioral and physiological effects of nicotine, it is also important to consider the consequences of long-term nicotine exposure on nAChR activity because nicotine levels remain chronically elevated at concentrations ranging from 10-300 nM with regular tobacco use (Benowitz et al., 1989). Chronic exposure to nicotine at tobacco-related levels causes a slow and reversible decrease in the ability of some nAChRs to be activated: a phenomenon referred to as desensitization (Boyd, 1987). Chronic nicotine also causes an increase in the number of some nAChR subtypes (Marks et al., 1983; Benwell et al., 1988; Flores et al., 1992, 1997). It has been suggested that a desensitized conformation serves as a "trigger" for this upregulation of receptor number (Marks et al., 1985; Schwartz and Kellar, 1985). By altering the number of activatable nAChRs, desensitization and upregulation are thought to underlie nicotine tolerance and sensitivity, as well as withdrawal symptoms associated with smoking cessation (Dani and Heinemann, 1996). Clearly, our understanding of nicotine's addictive properties would benefit from knowledge regarding the concentration ranges over which prolonged nicotine induces desensitization of different nAChR subtypes (Dani and Heinemann, 1996). An additional consideration is that nAChR desensitization may be regulated by physiological factors including Ca^{2+} and/or intracellular second messengers. The influence of such forms of regulation on receptor desensitization may significantly influence the outcome of prolonged nicotine on nAChR function and number. In an effort to understand the effects of nicotine-induced desensitization on receptor function and number, as well as the cellular factors that influence desensitization, the studies presented in this dissertation were undertaken to investigate the following relevant hypotheses: (1) nAChR desensitization is influenced by subunit composition; (2) desensitization and upregulation in the number of surface nAChRs are related phenomena; and (3) nAChR desensitization is influenced by extracellular Ca^{2+} and protein kinase C (PKC).

The remainder of this introduction will provide background information necessary for understanding the importance of these investigations (which are presented in the body

of the dissertation). An overview of the following will be included in this introduction: (1) nAChR diversity, (2) the use of heterologous expression systems to explore receptor function, (3) activation and desensitization of nAChRs, 4) upregulation of receptor number with chronic nicotine exposure, and (5) regulation of nAChR function by extracellular Ca^{2-} and kinase/phosphatase activity.

Diversity of nAChR Subtypes in the CNS

A systematic characterization of the concentration ranges over which nicotine interacts with the various conformational states (i.e., activatable and desensitized states) of nAChRs would greatly benefit our understanding of nicotine's action (Dani and Heinemann, 1996). However, this is not a straightforward task because a large (and currently unknown) number of functionally distinct nAChR subtypes exist in the CNS (see Role, 1992). To date, eleven neuronal subunits have been identified which include α2–α9 and β2–β4 (Boulter et al., 1987; Goldman et al, 1987; Duvoisin et al., 1989; see review Sargent, 1993). In heterologous expression systems, neuronal nAChRs can be formed from the pentameric arrangement (see Fig. 1) of two "agonist binding" α2–α4 subunits (α5 and α6 require expression of an additional α subunit species) and three "structural" β2–β4 subunits (Anand et al., 1991; Cooper et al., 1991). α7-α9 subunits can form functional nAChRs as homo-pentamers (Anand et al., 1993). Based on the known neuronal subunits, over a thousand structurally distinct nAChR subtypes are possible (see McGehee and Role, 1995). In addition, each individual nAChR subunit contributes unique properties to overall receptor function (Luetje and Patrick, 1991; Papke and Heinemann, 1991; Cachelin and Jaggi, 1991). Therefore, structural diversity accounts, in part, for the functional diversity of brain nAChRs (see McGehee and Role, 1995). Individual nAChR subunits exhibit unique and often overlapping distribution patterns. For example, a combination of *in situ* hybridization, PCR and immunocytochemistry studies reveal that α7, α4 and β2 nAChR subunits are expressed throughout rat brain at

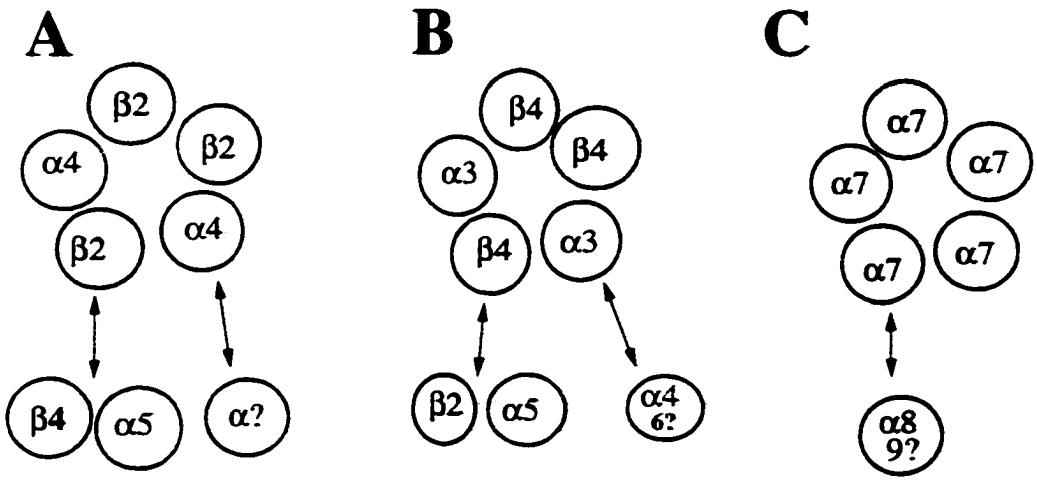


Figure 1. Structure of neuronal nAChRs. In *Xenopus* oocytes, α7, α8, and α9 subunits can form functional homomeric nAChRs; whereas the α2, α3, and α4 subunits require coexpression of either β2 or β4 subunits to form functional heteromeric nAChRs. The α5 subunit can be functionally incorporated into these heteromeric nAChR subtypes (Sargent, 1993; Lindstrom, 1997). *A.* The "high affinity" nAChR subtype found in the CNS contains α4 and β2 subunits (Flores et al., 1992; Piccioto et al., 1998). Some α4β2-containing nAChRs may also contain other subunits. For example, α4β2α5-containing and α4β2α3β4-containing nAChRs have been immunoisolated from neurons (Conroy and Berg, 1995). However, these are likely to represent a minority of total brain α4β2 nAChRs (Conroy and Berg, 1995). *B.* α3-containing nAChRs are expressed primarily in peripheral ganglia (Conroy and Berg, 1995, Ramirez-Latorre et al., 1996) and to a lesser extent in the CNS. A majority of the α3β4 and/or α3β2 containing nAChRs in autonomic ganglia (and perhaps in the CNS) are thought to also contain the α5 subunit (Conroy et al., 1992; Vernalis et al., 1993). *C.* α7-containing nAChRs are found both in the PNS and in the CNS. Cloned α7, α8 and α9 subunits can form functional homomeric nAChRs (Anand et al., 1993; Elgoyhen et al., 1994). In chick brain, heteromeric nAChRs containing α7 and α8 subunits have been identified (Keyser et al., 1993). Other hetomeric nAChR species may exist in the brain although they are probably expressed less abundantly.

high levels relative to α3 and α5 subunits, whereas α6, β3 and β4 subunits are found only in certain brain structures (Wada et al., 1989; Whiting et al., 1991). Because of the overlapping distribution of subunits (i.e., a single neuron may express mRNA for several subunits), subunit expression patterns do not simplify the issue of nAChR diversity (see McGehee and Role, 1995). Although nAChR diversity makes it difficult to systematically characterize brain nAChR function, this diversity may allow for the discovery of drugs that selectively interact with certain nAChR subtypes to elicit specific therapeutic effects.

Although the number of structurally/functionally distinct subtypes that exist in brain is not known, there are at least three major CNS nAChR subtypes for which partial subunit composition is known (see Fig. 1): (1) receptors that are sensitive to blockade by the snake venom α-bungarotoxin (α-BgTx), which contain α7 subunits; (2) receptors that bind nicotine with high affinity, which contain α4 and β2 subunits; and (3) receptors that bind nicotine with low affinity, which contain α3 and β4 or β2 subunits. It is important to note that in brain, it appears that subunits are promiscuous in their choice of partners for receptor assembly (Conroy et al., 1992; Anand et al., 1993; Ramirez-Latorre et al., 1996; Wang et al., 1996). Therefore, these are only partial subunit compositions, and receptors within each of these three general subtypes may contain additional subunits. The following paragraphs summarize some of what has been learned regarding the expression patterns and physiological properties of these major brain nAChR subtypes.

α4-containing nAChRs. One of the most abundant nAChR subtypes found in the CNS, which accounts for a majority of high-affinity [^{3}H]nicotine binding sites, are nAChRs that contain both α4 and β2 subunits (Whiting and Lindstrom, 1988; Flores et al., 1992; Picciotto et al., 1998). Recent studies of mutant mice that lack the α4 subunit indicate that α4β2-containing nAChRs may be involved in nociception (Marubio et al., 1999). Studies of knockout mice that lack the β2 subunit suggest that β2-containing nAChRs (which are likely to also contain α4 subunits) underlie, in part, the reinforcing

properties of nicotine. For these mice, nicotine self-administration is attenuated, and neurons in the "central reward" dopaminergic pathway no longer respond to nicotine (Picciotto et al., 1998). These mice also lack high-affinity nicotine binding sites ($\alpha4\beta2$ subunit-containing nAChRs). Functional expression of $\alpha4$ nAChRs in *Xenopus* oocytes requires coexpression of either $\beta2$ or $\beta4$ subunits (Anand et al., 1991; Cooper et al., 1991). Relative to other receptor subtypes that do not contain the $\alpha4$ subunit, heterologously expressed $\alpha4\beta2$ nAChRs display high affinities for nicotine binding and activation (Peng et al., 1994a; Vibat et al, 1995; Gopalakrishnan et al., 1996). Half-maximal activation of $\alpha4\beta2$ receptors has been estimated to occur between 0.1 and 10 μM nicotine (Bertrand et al., 1990; Hussy et al., 1994; Vibat et al., 1995; Buisson et al., 1996; Gopalakrishnan et al., 1996). Chronic exposure to low tobacco-related concentrations of nicotine leads to dramatic increases (greater than two fold) in the number of $\alpha4\beta2$ nAChRs both in brain and in heterologous expression systems (Flores et al., 1992; Peng et al., 1994a; Breese et al., 1997; Gopalakrishnan et al., 1997).

$\alpha7$-containing nAChRs. Another major nAChR subtype that is found both in the CNS and the peripheral nervous system (PNS) are $\alpha7$-containing nAChRs. Heterologous expression studies have revealed that $\alpha7$ (and $\alpha8$ or $\alpha9$) subunits can form functional homomeric nAChRs (Anand et al., 1993; Couturier et al., 1990). In brain, however, there is evidence that $\alpha7$ subunits, in addition to forming homomeric receptors, may also combine with $\alpha8$ (and possibly other subunits) to form nAChRs (Anand et al., 1993; Keyser et al., 1993). $\alpha7$-containing nAChRs have been shown to enhance the release of glutamate in the hippocampus (Gray et al., 1996) and in the medial habenula (McGehee et al., 1995). $\alpha7$-containing nAChRs are thought to play an important role in development because their activation appears to be critical for neuronal outgrowth and synaptogenesis (Chan and Quik, 1993; Role and Berg, 1996). Relative to other nAChR subtypes, $\alpha7$-containing nAChRs are highly permeable to Ca^{2+} ($pCa^{2+}/pNa^{+} > 15$),

display rapid desensitization kinetics, and are antagonized by and bind α-BgTx with high affinity (Couturier et al., 1990; Seguela et al., 1993). The high Ca^{2+} permeability of this receptor subtype may underlie its ability to modulate both neurotransmitter release and early gene expression during neural development. Half-maximal activation of heterologously expressed α7 receptors has been estimated to occur between 30 and 80 μM nicotine (Gopalakrishnan et al., 1995; Seguela et al., 1993; Peng et al., 1994b). Like other nAChR subtypes, α7 nAChRs in brain and in SH-SY5Y cells (a human neuroblastoma cell line) are upregulated in number by chronic nicotine exposure (Peng et al., 1997; Marks et al., 1983). However, much higher concentrations of nicotine than those obtained with tobacco use are required for upregulation of α7 receptors.

α 3-containing nAChRs. Like α4-containing nAChRs, functional expression of α3-containing nAChRs in *Xenopus* oocytes requires coexpression of either β2 or β4 subunits (Leutje and Patrick, 1991; Role, 1992). α3-containing nAChRs in brain and in autonomic ganglia may be composed of α3 subunits together with β4, β2, and α5 subunits (Vernalis et al., 1993; Ramirez-Latorre et al., 1996). α3-containing nAChRs are the predominant subtype in autonomic ganglia, where they mediate fast excitatory postsynaptic neurotransmission (Vernalis et al., 1993). In brain, α3-containing nAChRs are less abundant and have been implicated in presynaptic modulation of neurotransmitter release (Wonnacott et al., 1997; Sargent 1993). Studies of α3 knockout mice suggest that this an essential subunit of nAChRs required for normal function of the autonomic nervous system. The poor health of the α3 knockout mice precluded meaningful behavioral studies regarding the role of the α3 subunit in normal brain neurotransmission (Xu et al., 1999). In expression systems, half-maximal activation of α3β4 and α3β2 receptors occurs between 50 and 120 μM nicotine (Cohen et al., 1995; Wong et al., 1995; Wang et al., 1996). α3-containing nAChRs may also be upregulated with chronic

nicotine exposure but require higher concentrations than α4β2 nAChRs (Peng et al., 1997; Ke et al., 1998; Warpman et al., 1998).

The physiological role of subunit diversity. Despite extensive research characterizing functional differences of both expressed nAChR subtypes and native nAChRs (including pharmacological profiles, single channel properties, ion conductance, etc.), the physiological role served by diverse nAChR subtypes is poorly understood. One hypothesis is that structural differences between nAChR subtypes may allow for targeting of different receptor subtypes to distinct cellular compartments (i.e., preterminal, perisynaptic, somatodendritic regions). Consistent with this idea, it has been demonstrated that, when an intracellular loop of the α7 subunit is substituted for that of the α3 subunit, the α7 subunit (normally located perisynaptically) is then targeted to the presynaptic membrane, i.e., where the α3 subunit is generally located (Williams et al., 1998). Functional diversity might serve to fill unique functional roles required of receptors within these subcellular compartments (see Role and Berg, 1996; Colquhoun and Patrick, 1997). For example, it is possible that the high Ca^{2+} permeability of these perisynaptically located α7 nAChRs serves an important function in modulating intracellular Ca^{2+}-dependent processes (see Role and Berg, 1996; Williams et al., 1998). It has also been suggested that nAChR diversity serves to fill unique roles within different brain nuclei (see McGehee and Role, 1995; Colquhoun and Patrick, 1997). Consistent with this idea, *in situ* hybridization, pharmacological profiles, and radiolabeled ligand binding assays reveal a nonuniform distribution of nAChRs subunits throughout the brain (Wada et al., 1989; Marks et al., 1992; Clark, 1993; Seguela et al., 1993; Colquhoun and Patrick, 1997). Despite evidence that nAChR subtypes are differentially localized within cellular compartments and specific brain regions, the unique physiological roles of different nAChR subtypes remains to be determined.

Heterologous Expression of Neuronal nAChRs

An understanding of the physiological role of functionally diverse nAChR subtypes would benefit from knowledge regarding the subunit composition of native nAChRs and how subunit composition influences overall receptor function. *In situ* hybridization, PCR, and immunocytochemistry have provided some insight into the subunit composition of brain nAChRs. The weakness of these approaches is that a single neuron often expresses multiple subunits, but while the functional receptor pool is composed of only a small subset of these subunits (see Role and Berg, 1996; Colquhoun and Patrick, 1997). Therefore, much of what has been learned regarding the subunit composition of brain nAChRs has been obtained by comparing the functional properties of brain nAChRs to that of expressed receptors of known subunit composition (see Role, 1992; McGehee and Role, 1995). In addition, characterization of expressed nAChRs can provide insight regarding functional properties of native nAChRs for which at least partial subunit composition is known.

The *Xenopus* oocyte expression system has provided a convenient tool for examining protein function, including the function of nAChRs. This technique involves injection of RNA transcribed from cDNA clones that encode specific nAChR subunits. This RNA is then translated into subunit proteins that are inserted on the membrane as functional nAChRs. Functional data obtained from this technique have provided valuable insight into the properties of different nAChR subtypes and the influence of specific subunits on overall receptor function. For example, oocyte expression studies have revealed that cytisine is an effective agonist on β4-containing nAChRs but is much less effective on β2-containing nAChRs (Leutje and Patrick, 1991). The efficacy of cytisine for activation of nAChRs in the habenula, interpeduncular nucleus, and supra-chiasmatic ganglia (Mulle et al., 1991; Colquhoun and Patrick, 1997) is consistent with the β4 subunit being a major component of these nAChRs. Another example where oocytes have been useful in identifying subunit composition of native nAChRs is that of α7

nAChRs. Relative to other expressed nAChR subtypes, α7 nAChRs desensitize rapidly and bind α-BgTx with high affinity (Couturier et al., 1990; Seguela et al., 1993), thus solving the mystery of α-BgTx binding sites in brain, whose function, until recently, had been missed because of rapid desensitization (see Role, 1992).

Oocytes have also provided a useful tool for examining nAChR function; however, it does have some limitations. Although similarities exist between the pharmacological and biophysical properties of expressed and native nAChRs, there are few cases were the properties are exactly the same (McGehee and Role, 1995; Colquhoun and Patrick, 1997). An extensive comparison of the properties of expressed and native nAChRs by McGehee and Role (1995) can be summarized as follows: (1) Native nAChRs typically exhibit single channel conductance's that are 10-15 pS larger than that of nAChRs in oocytes; (2) native nAChRs have a greater mean open duration; and (3) despite some functional similarities, there are no exact matches between functional properties of expressed and native nAChRs.

Despite these limitations, until recently, the *Xenopus* oocyte expression system was the only system in which subunit composition of nAChRs could be examined. More recently, human embryonic kidney (HEK) cells have been utilized to express different nAChR subtypes. At the onset of the investigations presented in this dissertation, HEK cells were just beginning to be utilized for the investigation of expressed nAChRs. Table 1 provides a comparison of some of the functional properties of nAChRs expressed in HEK cells and in *Xenopus* oocytes. Although many of the pharmacological properties are the same, desensitization rates observed for nAChRs expressed in oocytes are much slower (10- to 100- fold) than those of nAChRs in HEK cells (see Table 1). This suggests that nAChR kinetics are regulated by the cellular environment and that oocytes may not be an ideal system to investigate nAChR kinetics (Lester and Dani, 1995; Wang et al., 1998). However, the relative influence of specific subunits on desensitization kinetics appears to be independent of the receptor's host cell (see Table 1).

Table 1. Comparison between nAChRs expressed in *Xenopus* oocytes and HEK cells

Subunit combination	α3β4				α4β2			
Expression System	HEK cells		oocytes		HEK cells		oocytes	
Species	rat	human	rat	human	rat	human	rat	human
γ (pS)			22			46	13&22	17&28
EC_{50} Ach (μM)	57	79	30	203	80	3	34	68
EC_{50} Nic (μM)	22	56	62	80	3	1.6	15	5.5
τ des fast (sec)	-10*	−1-5*	−200*		−180ms	−100ms	−5.4	
τ des fast (sec)					−2		−90	
Agonist potency profile			C>N=A	D>C=N >A	A>N	N>A>C	A=N> C	A>N >D>C

Subunit combination	α3β2				α7			
Expression system	HEK cells		oocytes		HEK cells		oocytes	
Species	rat	human	rat	human	rat	human	rat	human
γ (pS)			15					
EC_{50} Ach (μM)	370	121	350	440	300			180
EC_{50} Nic (μM)	45	83	123	132	120	49	90	113
τ des fast (sec)		−50ms	−8				−1.4	
τ des slow (sec)		−2	−300					
Agonist potency profile		N>A>C	D>A>N >C	D>A=N >C			N>C>D >A	D>C=A =N

Notes: C = cytisine, N = nicotine, A = acetylcholine, D = DMPP, * the fast phase was the dominant component of desensitization. References: Boulter et al., 1987; Cachelin and Jaggi, 1991; Luetje and Patrick, 1991; Papke and Heinemann, 1991; Seguela et al., 1993; Cachelin and Rust , 1995; Wong, 1995; Vibat et al., 1995; Buisson et al., 1996; Wang et al., 1996; Stauderman et al., 1998; Wang et al., 1998; Zhang et al., 1999.

Activation and Desensitization of Different Neuronal nAChR Subtypes

Because a variety of behavioral effects have been attributed to nicotine, nAChRs, as the molecular targets of nicotine, clearly serve an important role in modulating behaviors that are influenced by nicotine. The nAChR subtypes that modulate these behaviors can be determined if we know how nicotine effects the overall activity of different nAChR subtypes. Therefore, an important goal will be to determine and compare the concentration ranges over which nicotine activates and desensitizes different nAChR subtypes. The following paragraphs summarize some of what is known regarding nicotine-induced activation and desensitization and the rational for conducting a thorough investigation of the subject.

Although several researchers have examined nicotine-induced activation of different heterologously expressed nAChR subtypes, it is important to note that estimates of the nicotine concentrations that induce half-maximal (EC_{50}) activation of nAChRs vary widely between researchers. For example, estimates of the EC_{50} for nicotine-induced activation of heterologously expressed human $\alpha4\beta2$ nAChRs in HEK cells range 1.6 μM to 16 μM (Buisson et al., 1996; Gopalakrishnan et al., 1997). The reason for such varied estimates of a receptor's sensitivity to nicotine is that the functional properties of nAChRs may vary between receptor species (i.e., rat versus chick), expression systems (i.e., human embryonic kidney cells versus oocytes), and experimental techniques (i.e., rubidium efflux assays versus current amplitudes). For the example above, estimates of the EC_{50} values for nicotine–induced activation were obtained using whole-cell current amplitudes and a Rb^{2+}-efflux assay. Therefore, experimental parameters must be the same to provide an accurate comparison of nicotine activation (as well as desensitization, upregulation, and equilibrium binding). In summary, although nicotine activation has been examined for several expressed receptor subtypes, a systematic characterization of nicotine activation for the major nAChR subtypes under the same experimental conditions is lacking.

Whereas acute exposure to tobacco-related levels of nicotine may cause activation of some nAChRs, the continued presence of low, subactivating levels of nicotine (as occurs with nicotine abuse) may cause nAChRs to enter desensitized conformational states (Katz and Thesleff, 1957). Despite the relevance of desensitization to tobacco use, there has been relatively little research directed toward characterization of functional nAChR desensitization induced by prolonged low-level nicotine exposure. This is likely because agonist-induced desensitization is believed, by some, to be irrelevant to normal synaptic transmission (i.e., receptors are likely only briefly exposed to acetylcholine, during which time little desensitization is thought to occur) (see Jones and Westbrook, 1996). However, desensitized states are very relevant to tobacco use because they are thought to directly underlie and/or promote further cellular processes that result in nicotine tolerance and dependence (Dani and Heinemann, 1996). In addition to characterization of nAChR activation by nicotine, our understanding of nicotine's actions at the receptor level would also benefit from a systematic characterization of the concentration dependencies and kinetics of nicotine desensitization. An accurate comparison of the kinetics and the concentration dependencies of the major different nAChR subtypes for nicotine activation and desensitization could also be used to determine the unique contributions of a specific subunit to overall receptor function (see Cachelin and Jaggi, 1991; Cachelin and Rust 1995; Cohen et al., 1995). For example, to determine the functional receptor properties contributed by β2 subunit, we can compare α3β2 and α3β4 nAChRs or α4β2 and α4β4 nAChRs. Although the subunit composition of nAChRs in brain is unknown and many receptors may contain more that two different subunit types, many expressed receptors share the same properties as nAChRs from brain. The advantage of being able to provide accurate information regarding the contribution of a specific subunit to overall receptor function is that predictions can be made regarding native nAChR function even with the identification of only one subunit.

The goal, therefore, of the studies presented in the first article of this dissertation, "Influence of Subunit Composition on Neuronal Acetylcholine Receptors at Low Concentrations of Nicotine" (Fenster et al., 1997), is to systematically characterize both activation and functional desensitization induced by prolonged nicotine exposure for several nAChR subtypes, specifically, α7, α3β4, α4β4, α3β2, and α4β2 nAChRs. As stated above, to provide an accurate functional comparison of different nAChRs subtypes requires that all experiments be performed using the same receptor species clone, expression system, and experimental technique. In these studies, therefore, all experiments were performed on rat nAChRs expressed in *Xenopus* oocytes using a whole-cell two-electrode voltage-clamp technique. Kinetics and affinities for activation and desensitization in response to activating doses of nicotine can be measured and characterized directly from whole-cell current amplitudes. Desensitization and recovery from desensitization induced by low (tobacco-related) subactivating levels of nicotine can be measured using a modified version of the experimental design described by Katz and Thesleff (1957). For these experiments, the desensitizing dose of agonist was continually applied and changes of receptor function were assayed using brief non-desensitizing test-pulses of agonist administered before, during, and after desensitizing agonist exposure. To determine the affinity of the different nAChR subtypes for nicotine desensitization, dose-response curves can be constructed from the relative magnitudes of steady state desensitization induced by different concentrations of nicotine.

A comparison of the different nAChR subtypes' affinities for nicotine activation and desensitization will allow for predictions to be made regarding the outcome of prolonged exposure to nicotine (at tobacco-related levels) on receptor function. In addition, a systematic characterization of the rates at which these receptors return to activatable states after nicotine desensitization will allow for predictions to be made regarding the outcome of nicotine exposure on nAChR function once nicotine is removed. These data provide information that is relevant to the effects of tobacco use on

the function of different brain nAChR subtypes. For example, as nicotine levels gradually fall after tobacco use (the half-life of nicotine in the blood stream is about 2 hrs), receptors may recover from desensitization. The rates at which they recover will influence nAChR activity (Dani and Heinemann, 1996).

Neuronal type nAChRs are highly permeable to Ca^{2-} (Mulle et al., 1992a; Vernino et al., 1992; Seguela et al., 1993), and the function of some nAChR subtypes can be modulated by physiologically relevant changes in extracellular Ca^{2-} (Mulle et al.1992b; Amador and Dani, 1995; Galzi et al., 1996). One way that changes in extracellular Ca^{2-} can modulate nAChR function is through a direct interaction of Ca^{2-} with binding sites on the receptor (Galzi et al., 1996). For example, the efficacy of agonist for activation of α7 homomeric nAChRs is enhanced by a direct interaction of extracellular Ca^{2-} with external binding sites on α7 subunits (Galzi et al., 1996). In addition to direct binding, Ca^{2-} influx can activate Ca^{2-}-dependent intracellular second messengers that, in turn, modulate nAChR function (Mulle et al., 1992b; Hardwick and Parsons, 1996; Khiroug et al., 1997). Therefore, it was necessary that the experiments in this study be performed both in the presence of extracellular Ca^{2-} and under "Ca^{2-}-free" conditions. Experiments which systematically investigate the effects of extracellular Ca^{2-} on the function of different nAChR subtypes will lay the ground work for studies directed toward understanding the mechanism underlying Ca^{2-} modulation of nAChR function.

Upregulation of nAChR Number After Chronic Nicotine Exposure

Heterologous expression studies reveal that α4β2 receptors have high affinities for nicotine binding and activation relative to other (non-α4-containing) nAChR subtypes (Hussy et al., 1994; Vibat et al., 1995). The high affinity of α4β2 subunit-containing nAChRs makes these receptors a likely target for mediating the physiological effects of low, tobacco-related levels of nicotine (< 1 μM nicotine; Benowitz et al., 1989). For example, long-term exposure to low (tobacco-related) levels of nicotine induces an

upregulation in the number of high-affinity [^{3}H]nicotine binding sites in the CNS, a vast majority of which are $\alpha4\beta2$ subunit-containing nAChRs (Marks et al., 1983; Benwell et al., 1988; Flores et al., 1992). Chronic nicotine exposure is also associated with a down-regulation of $\alpha4\beta2$ receptor function (Marks et al., 1993; Peng et al., 1994a; Hsu et al., 1996). This decrease in receptor function is thought to be, in part, a consequence of agonist-induced desensitization (see Boyd, 1987). Because the major effects of prolonged nicotine exposure at tobacco-related concentrations are desensitization and upregulation of $\alpha4\beta2$ containing nAChRs, these processes are likely to contribute to the addictive properties of nicotine.

How chronic nicotine initiates upregulation of nAChR number remains speculative. Originally it was proposed that upregulation after chronic nicotine is an adaptive response of neurons to a decrease in nAChR function, presumably resulting from agonist-induced desensitization (Marks et al., 1985; Schwartz and Kellar, 1985). If this hypothesis is true, then antagonists (which inhibit receptor activation) should also be able to induce upregulation. In contrast to this prediction, it has been demonstrated that upregulation of nAChRs in brain is not induced by chlorisondamine, a noncompetitive antagonist that produces a long lasting (6 to 12 weeks) inhibition of receptor function (el-Bizri and Clark, 1994). In addition, nAChRs expressed in non-neuronal cells are upregulated by chronic nicotine exposure (Peng et al., 1994a; Bencherif et al., 1995; Gopalakrishnan et al., 1997), which suggests that upregulation is not a neuron-specific process. Another possibility is that upregulation is a consequence of receptor activation that occurs with chronic agonist exposure. However, upregulation of heterologously expressed $\alpha4\beta2$ receptors has been observed after chronic exposure to competitive antagonists that do not induce receptor activation (Peng et al., 1994a; Whiteaker et al., 1998). In summary, it is unlikely that upregulation results directly from changes in receptor function.

It has also been hypothesized that a desensitized receptor conformation serves as a "trigger" for upregulation of α4β2 nAChR number (Peng et al., 1994a; Marks et al., 1985; Schwartz and Kellar et al., 1985). If the desensitization hypothesis for upregulation of receptor number is correct (see Fig. 2), then nicotine concentrations that produce α4β2 nAChR desensitization should also induce upregulation. Based on this prediction, recent studies have concluded that the desensitization hypothesis for upregulation is false (Peng et al., 1994a; Whiteaker et al., 1998) because the concentrations of nicotine required to bind desensitized receptors and to induce upregulation differ by several orders of magnitude (Peng et al., 1994a; Bencherif et al, 1995; Whiteaker et al., 1998). These conclusions, however, are based on the following assumptions, which may not be entirely accurate. The first assumption is that the agonist receptor binding assays used for these studies accurately assess the desensitized state(s) of the receptor (see Fig. 2). Because dose-response relationships constructed from functional estimates of desensitization and from equilibrium binding assays can differ by several orders of magnitude (Marks et al., 1996), it is reasonable to conclude that the first assumption might not be valid. Another consideration is the methodology used by previous investigators to measure equilibrium binding affinities. Specifically, these researchers utilized a standard equilibrium [^{3}H]agonist binding technique which involves solubilizing the receptors from the membrane. The assumption here is that measurements of agonist binding in standard membrane homogenization assays accurately reflect measurements of agonist binding to receptors on intact cells.

It is possible, however, that membrane homogenization influences the conformational state of the receptor and/or provides binding measurements of surface receptors that are "contaminated" by intracellular receptors that exist in higher affinity states (Whiteaker et al., 1998). If these assumptions are not accurate, then it is possible that the desensitization hypothesis for upregulation of surface nAChRs is still valid. Therefore, both the validity of these assumptions and the desensitization hypothesis

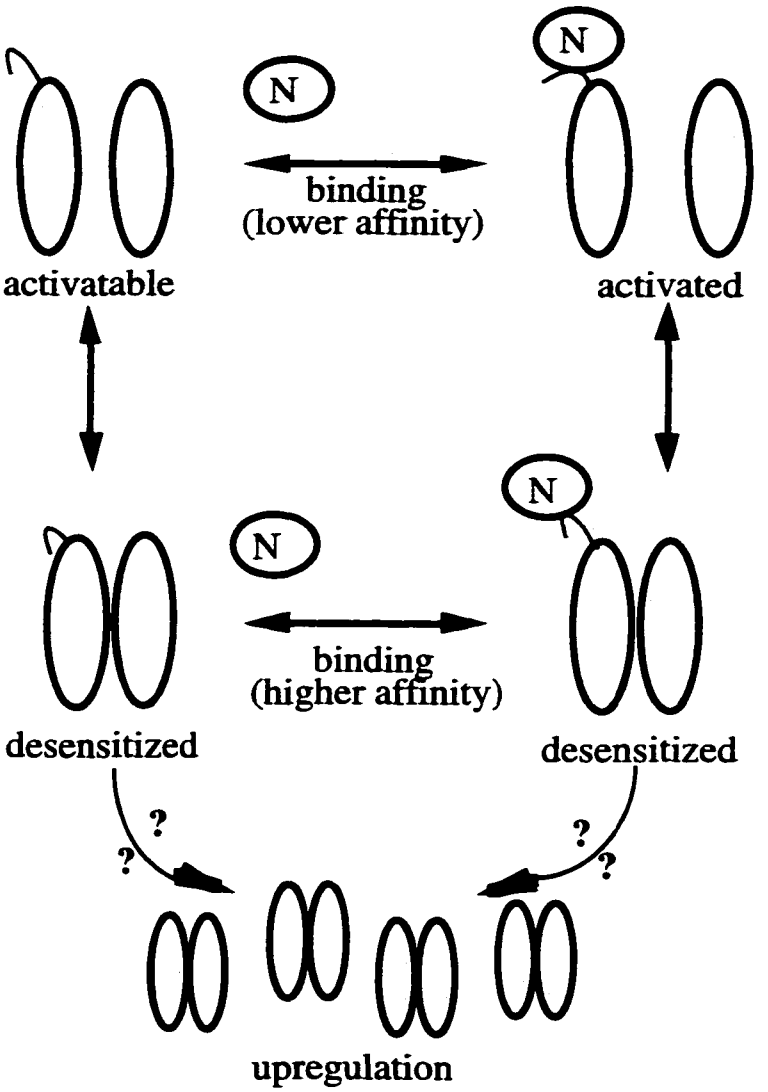


Figure 2. Desensitized nAChR states hypothetically serve as a "trigger" for upregulation of receptor number that occurs with chronic nicotine. Desensitized receptor states have a higher affinity for binding nicotine than activatable receptor states. Equilibrium [^{3}H]nicotine binding assays are thought to provide measure the binding affinity of the desensitized receptor state (Lippiello et al., 1987).

are re-examined in the second article of this dissertation, "Upregulation of Surface α4β2 Nicotinic Receptors Is Initiated by Receptor Desensitization after Chronic Exposure to Nicotine" (Fenster et al., 1999b).

If the desensitization hypothesis for upregulation of surface α4β2 nAChRs is correct, then it is predicted that nicotine concentrations that produce receptor desensitization should also induce receptor upregulation. The most direct way to test this prediction is to assess receptor desensitization functionally and then compare these results with receptor upregulation measured from equilibrium [^{3}H]nicotine binding assays to intact, cell-surface receptors. Because *Xenopus* oocytes are capable of expressing millions of functional nAChRs over their large surface area, these binding and physiological assays can be conveniently performed for α4β2 nAChRs expressed in oocytes. Dose-response curves for both functional desensitization and upregulation of receptor number induced by chronic nicotine can be constructed as follows: First, groups of oocytes are chronically exposed (1 to 2 days) to nicotine over a range of different concentrations; then, upregulation of surface nAChRs can be measured using a modified equilibrium [^{3}H]nicotine binding assay to intact oocytes. Functional desensitization can be measured using whole-cell current amplitudes, rather than indirectly from binding assays as in previous studies (Peng et al., 1994a; Bencherif et al., 1995; Whiteaker et al., 1998). Changes in receptor number and function that occur independently of chronic nicotine exposure can be determined from changes in parallel control oocytes not exposed to nicotine. If these estimates of the nicotine concentrations that produced half-maximal upregulation and functional desensitization are similar, then these data would support the desensitization hypothesis for receptor upregulation of surface α4β2 nAChRs.

Another way to test the desensitization hypothesis for upregulation of surface α4β2 nAChRs is to demonstrate that α4β2 nAChRs that fail to recover from desensitization will upregulate even after nicotine is removed. To test this prediction,

upregulation can be quantified for a mutant α4β2 nAChR that fails to recover fully from nicotine desensitization under conditions that, for wild-type receptors, allow for recovery. If experimental results are consistent with this prediction, then this demonstration would not only support the desensitization hypothesis, but would also suggest that (assuming that nicotine fully dissociates from the receptor) receptor occupation by nicotine is not a requirement for α4β2 nAChR upregulation.

In addition to upregulation of surface α4β2 nAChRs, other nAChR subtypes can also be upregulated by chronic nicotine exposure. For example, both in brain and in heterologous expression systems, upregulation of α3-containing and α7-containing nAChRs has been observed after chronic nicotine exposure. However, unlike α4β2 nAChRs, upregulation of these nAChR subtypes requires much higher nicotine concentrations than occur with tobacco use (Peng et al., 1997; Ke et al., 1998; Warpman et al., 1998). To further investigate the desensitization hypothesis and to see if a desensitized receptor state might serve as a common "trigger" for upregulation of other nAChRs subtypes, we can test the prediction that nAChRs with lower affinities for nicotine-induced functional desensitization will also exhibit lower affinities for upregulation. To test this prediction, estimates of the nicotine concentrations that produce half-maximal upregulation and functional desensitization can be obtained for a receptor subtype with lower affinity for nicotine desensitization by using the same experimental procedures described above. The results from these experiments should provide additional insight regarding the relationship between functional desensitization and upregulation of surface nAChRs.

Several previous studies have concluded that desensitization and upregulation are unrelated processes (Peng et al., 1994a; Benchrerif et al., 1995; Whiteaker et al., 1998). This conclusion, however, is based on several assumptions. Specifically, these studies formed their conclusions based on the findings that, for expressed α4β2 nAChRs, apparent affinities for agonist-induced upregulation differ by orders of magnitude from

equilibrium [^{3}H]agonist binding to standard membrane preparations. One of the assumptions is that agonist equilibrium binding assays provide accurate measurements of nAChRs in their desensitized state(s). If this is a valid assumption, then equilibrium [^{3}H]nicotine binding and nicotine-induced functional desensitization should exhibit similar dose-dependencies. To test this prediction, dose-response curves can be constructed for equilibrium [^{3}H]nicotine binding to α4β2 nAChRs on intact oocytes from which we can attain an estimate of the concentration of nicotine required for half-maximal (EC_{50}) equilibrium binding. However, to measure the affinity of the desensitized state for agonist, previous studies used membrane homogenates to perform agonist equilibrium binding assays. The assumption here is that membrane homogenization does not alter the desensitized state(s) of the receptor as it exists on the surface of intact cells. If this assumption is correct, then dose-dependencies of equilibrium [^{3}H]nicotine binding to receptors from membrane homogenates should be similar to those of both functional nicotine-induced desensitization and equilibrium [^{3}H]nicotine binding to surface receptors in intact preparations. To test this prediction, dose-response curves can be constructed for equilibrium [^{3}H]nicotine binding to α4β2 nAChRs from membrane homogenates and compared to those constructed for equilibrium [^{3}H]nicotine binding to α4β2 nAChRs on intact cells. A shift in the dose-response curve after membrane homogenization might arise from the existence of internal receptors with different affinities for binding agonist (Wonnacott, 1987). For example, for α4β2 nAChRs expressed in M10 cells, greater than 80% of the receptors are intracellular (Whiteaker et al., 1998). In addition, apparent affinities for [^{3}H]agonist equilibrium binding to α4β2 nAChRs in intact M10 cells were shifted to the left after membrane homogenization. This shift was greater for cell-impermeant [^{3}H]agonists such as acetylcholine (Whiteaker et al., 1998). These results are consistent with the possibility that intracellular α4β2 nAChRs exist in higher affinity states than surface α4β2 nAChRs receptors. An alternative explanation is that membrane homogenization disrupts the

normal biochemical state of both intracellular and surface nAChRs and that this causes a shift in apparent agonist binding affinities. The results from these experiments should provide further insight into the relationship between functional desensitization and equilibrium binding to intact cells versus membrane homogenates.

Regulation of nAChR Desensitization by Calcium and Protein Kinase C

If desensitization initiates upregulation of receptor number that occurs with chronic nicotine exposure (Wonnacott, 1990; Schwartz and Kellar, 1985), then factors that modulate nAChR desensitization may contribute to the long-term effects of nicotine on nAChR number and function. Desensitization is a common feature of ligand-gated ion channels that is subject, in many cases, to modulation by intracellular second messengers (Jones and Westbrook, 1996; see Smart, 1997). For example, in the case of muscle-type nAChRs, a variety of studies both *in vivo* and *in vitro* reveal that rates of desensitization are enhanced by cAMP-dependent protein kinase (PKA) phosphorylation (Huganir and Greengard, 1983; Huganir et al., 1986; Miles et al., 1987; Hoffman et al., 1994). There is also evidence that kinase activity also regulates desensitization of neuronal-type nAChRs. For example, studies of neuronal nAChRs reveal that PKC enhances desensitization of nAChRs in sympathetic ganglia nAChR (Downing and Role, 1987) and that recovery from inactivation is enhanced by PKC activity for heterologously expressed neuronal nAChRs (Eilers et al., 1997). Physiological changes in extracellular Ca^{2+} concentrations may also modulate nAChR function (Mulle et al., 1992b; Vernino et al., 1992; Amador and Dani, 1995; Galzi et al., 1996). For example, the potentiation of the response of nAChRs from isolated habenular neurons occurs with increases in extracellular Ca^{2+} concentration (Mulle et al., 1992b). This potentiating effect is due to a direct interaction of Ca^{2+} with external sites on the receptor (Mulle et al., 1992b; Galzi et al., 1996). Early studies of nAChR desensitization showed that extracellular Ca^{2+} accelerates desensitization onset (Manthey et al., 1966). More recent studies suggest that this effect is

due to elevations in intracellular Ca^{2+} levels that occur with nAChR activation (Miledi, 1980; Khiroug et al., 1997) and the subsequent activation of Ca^{2+}-dependent intracellular second messengers (Khiroug et al., 1998). Because the activity of several PKA and PKC isoforms are Ca^{2+}-dependent, it is possible that extracellular Ca^{2+} may play a similar role in the aforementioned examples of second messenger regulation of nAChR desensitization. It possible that activation of Ca^{2+}-dependent intracellular messengers influences receptor function by influencing the phosphorylation state of specific neuronal nAChR subunits (Vijayaraghavan et al., 1990; Nakayama et al., 1993; Moss et al., 1996; Viseshakul et al., 1998).

Previously it has been demonstrated that, for α4β2 nAChRs expressed in *Xenopus* oocytes, recovery from desensitization is dependent upon the presence of extracellular Ca^{2+}. These findings lend some support to the idea that Ca^{2+} influx through α4β2 nAChRs activates Ca^{2+}-dependent second messengers that either directly (through receptor phosphorylation) or indirectly modulate α4β2 nAChR function. The goal, therefore, of the studies presented in the third article of this dissertation, "Regulation of α4β2 Nicotinic Receptor Desensitization by Calcium and Protein Kinase C" (Fenster et al., 1999a), is to further investigate the role of extracellular Ca^{2+} and receptor phosphorylation on desensitization of α4β2 nAChRs.

If α4β2 nAChR desensitization is modulated by an interaction of Ca^{2+} with specific binding sites on the receptor, then Ba^{2+}, which can also bind to "Ca^{2+}" binding sites, should be able to in effect substitute for Ca^{2+} (Mulle et al., 1992b). Therefore, to further assess the role of extracellular Ca^{2+} in its regulation of α4β2 nAChR desensitization, we can examine the effects of replacing extracellular Ca^{2+} with Ba^{2+} on desensitization of α4β2 nAChRs expressed in *Xenopus* oocytes. Desensitization and recovery from desensitization can be examined using a modification of the "classic" protocol described by Katz and Thesleff (1957). Using a similar protocol, Feltz and Trautmann (1982) demonstrated that desensitization of muscle-type nAChRs is a biphasic

process with an initial fast phase and a second slower phase. From these experiments, therefore, we should be able to determine whether desensitization is a biphasic process for α4β2 nAChRs. A comparison of the data obtained in the presence of Ca^{2+} and in the presence of Ba^{2+} will allow us to determine if desensitization and recovery are modulated by a Ca^{2+}-dependent process that is Ba^{2+} insensitive. In addition, if desensitization is biphasic, then we will also be able to determine which rates are modulated.

For both muscle-type nAChRs (Hardwick and Parsons, 1996) and neuronal nAChRs in chromaffin cells (Khiroug et al., 1998), recovery from desensitization is influenced by the balance of PKC and Ca^{2+}-dependent phosphatase activity. It is possible that recovery from desensitization is similarly regulated for α4β2 nAChRs. To investigate this possibility, we can examine the effects of treating oocytes with drugs that modulate PKC and/or phosphatase activity on α4β2 nAChR desensitization and recovery. Oocytes expressing α4β2 nAChRs can be injected with either the activator of PKC, phorbol-12-myristate-13-acetate (PMA, or with calphostin-C (an inhibitor of PKC), and rates of desensitization and recovery from desensitization can be determined. Calcineurin is a Ca^{2+}-dependent phosphatase that has been shown to affect the function of many ligand-gated channels (Yakel, 1997). Therefore, to examine the effects of phophatase inhibition, we can inject oocytes expressing α4β2 nAChRs with cyclosporin-A (an inhibitor of calcineurin). If desensitization after treatment with cyclosporin-A is similar to that after treatment with PMA, one conclusion is that desensitization of α4β2 nAChRs is influenced by PKC and phophatase activity, possibly by altering the phosphorylation state of the receptor.

The α4 subunit contains five consensus sites for PKC-dependent phosphorylation on the cytoplasmic loop between the third and fourth transmembrane domains, and the β2 subunit contains one PKC site in this region (Goldman et al., 1987; Deneris et al., 1988). In addition, the α4 subunit of α4β2 nAChRs has been shown to be heavily phosphorylated *in vivo* (Viseshakul et al., 1998). It is therefore possible that α4β2

nAChRs are regulated by PKC-dependent receptor phosphorylation. To further examine this possibility, we can investigate the effects of mutating specific PKC phosphorylation consensus sites on α4β2 nAChR desensitization. The results of these experiments should elucidate the role of extracellular Ca^{2+} and/or second messengers on desensitization of α4β2 nAChRs. Because these studies examine the effects of Ca^{2+} and PKC on desensitization of α4β2 nAChRs induced by nicotine at levels associated with tobacco use, the results will also allow us to observe how these physiologically relevant forms of regulation can influence α4β2 nAChR function during tobacco use.

In summary, the overall goal of the studies presented in this dissertation is to enhance our understanding of the outcome of prolonged nicotine exposure on the number and function of different nAChR subtypes. In the first study, "Influence of Subunit Composition on Neuronal Acetylcholine Receptors at Low Concentrations of Nicotine," we provide a systematic characterization of affinities for nicotine-induced activation and steady-state functional desensitization for different nAChR subtypes expressed in *Xenopus* oocytes. From the results of these experiments, predictions can be made regarding the outcome of prolonged nicotine exposure on the function of different nAChR subtypes. The rates at which these nAChRs are desensitized and recover from desensitization are also characterized. From the results of these experiments, predictions can be made regarding the rates at which these receptors desensitize as nicotine levels rise (i.e., during tobacco use) and the rates at which they recovery as nicotine levels fall (i.e., after tobacco use). In the second study, "Upregulation of Surface α4β2 Nicotinic Receptors Is Initiated by Receptor Desensitization after Chronic Exposure to Nicotine," we examine the hypothesis that upregulation of α4β2 nAChR number that occurs with tobacco use is linked to receptor desensitization. The α4β2 nAChRs is the focus of these studies because the major known effects of chronic exposure to tobacco-related levels of nicotine in brain is to reduce the α4β2 nAChR function while at the same time upregulating α4β2 nAChR number. The desensitization hypothesis for upregulation will

also be examined for other receptor subtypes. If it can be demonstrated desensitized receptor states serve to "trigger" upregulation of nAChR number, this will be useful for predicting the long-term consequences of tobacco-related levels of nicotine on different subtypes of nAChRs in the CNS. In the third study, "Regulation of α4β2 Nicotinic Receptor Desensitization by Calcium and Protein Kinase C," we examine the influence of extracellular Ca^{2+} and PKC on α4β2 nAChR desensitization induced by prolonged low-level nicotine exposure. If it can demonstrated that Ca^{2+} and/or PKC influence α4β2 nAChR desensitization, this implies that physiologically relevant factors may influence the outcome of prolonged nicotine exposure on both nAChR function and number. The results of these studies and their implications for understanding nicotine addition at the receptor levels are discussed in the Summary and Conclusions.

INFLUENCE OF SUBUNIT COMPOSITION ON DESENSITIZATION OF NEURONAL ACETYLCHOLINE RECEPTORS AT LOW CONCENTRATIONS OF NICOTINE

by

CATHERINE P. FENSTER, M. FELICIA RAINS, BRETT NOERAGER, MICHAEL W. QUICK AND ROBIN A.J. LESTER

The Journal of Neuroscience (1997) 17:5747-5759

ABSTRACT

The influence of α and β subunits on the properties of nicotine-induced activation and desensitization of neuronal nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus* oocytes was examined. Receptors containing α4 subunits were more sensitive to activation by nicotine than α3-containing receptors. At low concentrations of nicotine, nAChRs containing β2 subunits reached near-maximal desensitization more rapidly than β4-containing receptors. The concentration of nicotine producing half-maximal desensitization was influenced by the particular α subunit expressed; similar to results for activation, α4-containing receptors were more sensitive to desensitizing levels of nicotine than α3-containing receptors. The α subunit also influenced the rate of recovery from desensitization; this rate was approximately inversely proportional to the apparent nicotine affinity for the desensitized state. The homomeric α7 receptor showed the lowest sensitivity to nicotine for both activation and desensitization; α7 nAChRs also demonstrated the fastest desensitization kinetics. These subunit-dependent properties remained in the presence of external calcium, although subtle, receptor subtype-specific effects on both the apparent affinities for activation and desensitization and desensitization kinetics were noted. These data imply that the subunit composition of various nAChRs will determine the degree to which receptors are desensitized and/or activated by tobacco-related levels of nicotine. The subtype-specific balance between receptor activation and desensitization should be considered important when the cellular and behavioral actions of nicotine are interpreted.

INTRODUCTION

Neuronal nicotinic receptors (nAChRs) are a functionally diverse group of ligand-gated ion channels formed from the pentameric arrangement of one or more individual subunits (Couturier et al., 1990; Anand et al., 1991; Cooper et al., 1991; Luetje and Patrick, 1991). The existence of multiple subtypes (Role, 1992), their nonuniform

distribution within the CNS (Duvoisin et al., 1989; Wada et al., 1989; Morris et al., 1990; Whiting et al., 1991; Dineley-Miller and Patrick, 1992; Segeula et al., 1993), and their localization to both pre- and postsynaptic zones (Clark, 1993; McGehee and Role, 1996; Role and Berg, 1996; Wonnacott, 1997) implies diverse functions of nAChRs and suggests that several mechanisms are involved in the behaviorally important actions of nicotine.

At a minimum, any cellular and molecular theory of nicotine dependency must take into account that nAChRs can be both activated and desensitized by nicotine (Katz and Thesleff, 1957). Much evidence supports the idea that each of these receptor states are critical in addiction (Stolerman and Shoaib, 1991; Dani and Heinemann, 1996). Because these receptor states vary according to subunit composition (Cachelin and Jaggi, 1991; Gross et al., 1991; Hsu et al., 1995; Vibat et al., 1995), subtypes of nAChRs may be involved differentially in the acute and chronic effects of nicotine and their associated cellular compensatory events (Balfour, 1994). These include subtype-specific increases in receptor number within various brain regions (Marks et al., 1992). Moreover, the extent to which nAChR subtypes are activated and desensitized by nicotine could determine whether specific receptor subtypes are functionally up- (Rowell and Wonnacott, 1990; Gopalakrishnan et al., 1996) or downregulated (Marks et al., 1993) during chronic agonist exposure (Hsu et al., 1995).

Although the actual subunit composition of native CNS nAChRs is unknown, many expressed receptor subtypes (Whiting and Lindstrom, 1988; Flores et al., 1992; Anand et al., 1993) share characteristics of CNS nAChRs. Thus, understanding the influences of different subunits on receptor function has value both for determining dominant subunits in native receptors and for predicting the effects of nicotine on nAChRs composed of particular subunits. Previous studies have addressed how subunit composition affects the time course of desensitization and the agonist potency for activation (Cachelin and Jaggi, 1991; Gross et al., 1991; Luetje and Patrick, 1991; Cohen

et al., 1994; Hsu et al., 1995; Vibat et al., 1995). However, many of these studies used acetylcholine as the principal agonist. Because agonists can differentially affect desensitization, it is not possible to predict accurately how nicotine will interact with desensitized states of nAChRs. Moreover, except for one report (Hsu et al., 1995), desensitization has not been characterized using low, tobacco-related concentrations of nicotine (Benowitz et al., 1989). In the present study, we evaluate the action of low concentrations of nicotine on both activation and desensitization of a number of nAChRs expressed in *Xenopus* oocytes and examine the contribution of both α and β subunits to these processes.

MATERIALS AND METHODS

Xenopus *Oocyte Preparation and cDNA Injection*

Procedures for preparation of oocytes have been described in detail elsewhere (Quick and Lester, 1994). Briefly, oocytes were defolliculated and maintained at 18°C in incubation medium containing ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4), 1.8 or 3.6 mM $CaCl_2$, 50 μg/ml gentamycin, and 5% horse serum. Subunit cRNAs were synthesized *in vitro* (Machine Message; Ambion Inc., Austin, TX) from linearized plasmid templates of rat cDNA clones. Oocytes were injected with between 5 and 25 ng/subunit/oocyte; α and β subunit cRNAs were injected in 1:1 ratios, although in some experiments in which α3β4 receptors were examined, cRNAs ratios of 10:1 α3:β4 and 1:10 α3:β4 were injected. All salts and drugs were obtained from Sigma (St. Louis, MO).

Electrophysiology

Whole-cell currents were measured at room temperature (20-25°C), 24-96 hr postinjection with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) in a standard two-microelectrode voltage-clamp configuration. Electrodes were filled with 3 M

KCl and had resistances of 0.5-2 MΩ. Oocytes were clamped between -40 and -60 mV and superfused continuously in calcium-free ND96 (i.e., nominally calcium-free solution) or in the presence of 3.6 mM calcium (i.e. calcium-containing solutions). This concentration of calcium is in the middle of the range for potentiation of nAChRs responses (Mulle et al., 1992b; Vernino et al., 1992). For comparison, the effects of 1.8 mM calcium and 3.6 mM calcium were examined on α7 receptors. All drugs were applied in these solutions. (-)-nicotine tartrate (nicotine) and acetylcholine chloride (acetylcholine) were prepared from frozen stock solutions (100 mM). Atropine (1 μM) was included in the superfusion solution to block endogenous muscarinic responses. All currents were recorded either on chart recorder and/or on an 80486-based PC using AxoScope software (Axon Instruments) after 50-100 Hz low-pass filtering at a digitization frequency of 200 Hz. For slowly desensitizing responses, peak currents were assessed on-line from the digital read-out of the amplifier and/or off-line using AxoScope software.

Peak currents at EC_{50} concentrations were typically in the range of 100 nA-2 μA. Currents as small as 5 nA could be measured, although for accuracy only currents above 20 nA were included in the desensitization measurements. Concentration-response curves were fit with logistic expressions to estimate the EC_{50} (activation) and IC_{50} (desensitization). Single and/or double exponential fits of the data were used to compare the time courses of desensitization and recovery. Exponential curves were fit to either the desensitizing phase of the nAChR responses or to the time course of desensitization development as assessed from the depression of test pulses applied during incubation with nicotine. In both cases exponentials were constrained not to fall below zero current. For some nAChR subtypes, recovery from desensitization could be slow (>1 hr) and often was incomplete before the recording became unstable. For these data the exponential fits were constrained to allow full recovery back to control values.

Solutions were gravity-fed via a six-way manual valve (Rainin Instruments, Woburn, MA) to the oocyte in the recording chamber; some solution mixing occurred in

the dead space between the valve and the chamber. To estimate the effect of dead space on the agonist application, we calculated the solution-exchange rate from the time course of inhibition of the current induced by nicotine (5 μM) through β4 subunit-containing receptors during a step from medium containing 96 mM NaCl to one containing 48 mM NaCl (96 mM sucrose was used to restore osmolality). The time constant for the exchange, obtained from a single exponential fit, was 1.3 ± 0.3 sec (10 observations from five different oocytes). Thus, solution exchange was complete within ~5 sec. Because of this noninstantaneous exchange, at high agonist concentrations and in particular for α7 nAChRs that desensitize very rapidly, true peak currents are underestimated. This may result in an overestimation of the EC_{50} for activation and a smaller Hill coefficient. Although this is a potential problem in certain types of analyses, in the present study we were concerned for the most part with activation and desensitization at relatively low agonist concentrations in which the influence of exchange rate is less critical. All data are expressed as the mean ± SEM. For statistical comparisons of concentration-response curves, *t* tests were performed on the regression coefficients, estimated using the method of probits (Finney, 1971). For statistical comparisons of mean data, weighted means *t* tests were performed for all unpaired comparisons.

RESULTS

Potency of Nicotine for Activation of nAChRs

The differential sensitivity of nicotine in activating various expressed receptors (Luetje and Patrick, 1991) implies that nicotine is not equipotent at all nAChRs. Nicotine-induced concentration-response relationships for four different expressed nAChRs (α3β4, α3β2, α4β4, and α4β2) confirms this hypothesis (Fig. 1). Logistic fits to these concentration-response curves estimate EC_{50} values that can be divided into two groups (Table 2): receptors containing α4 subunits have lower EC_{50} values than those containing α3 subunits ($t = 13.27$; $p < 0.01$), implying that α4 subunits confer a higher

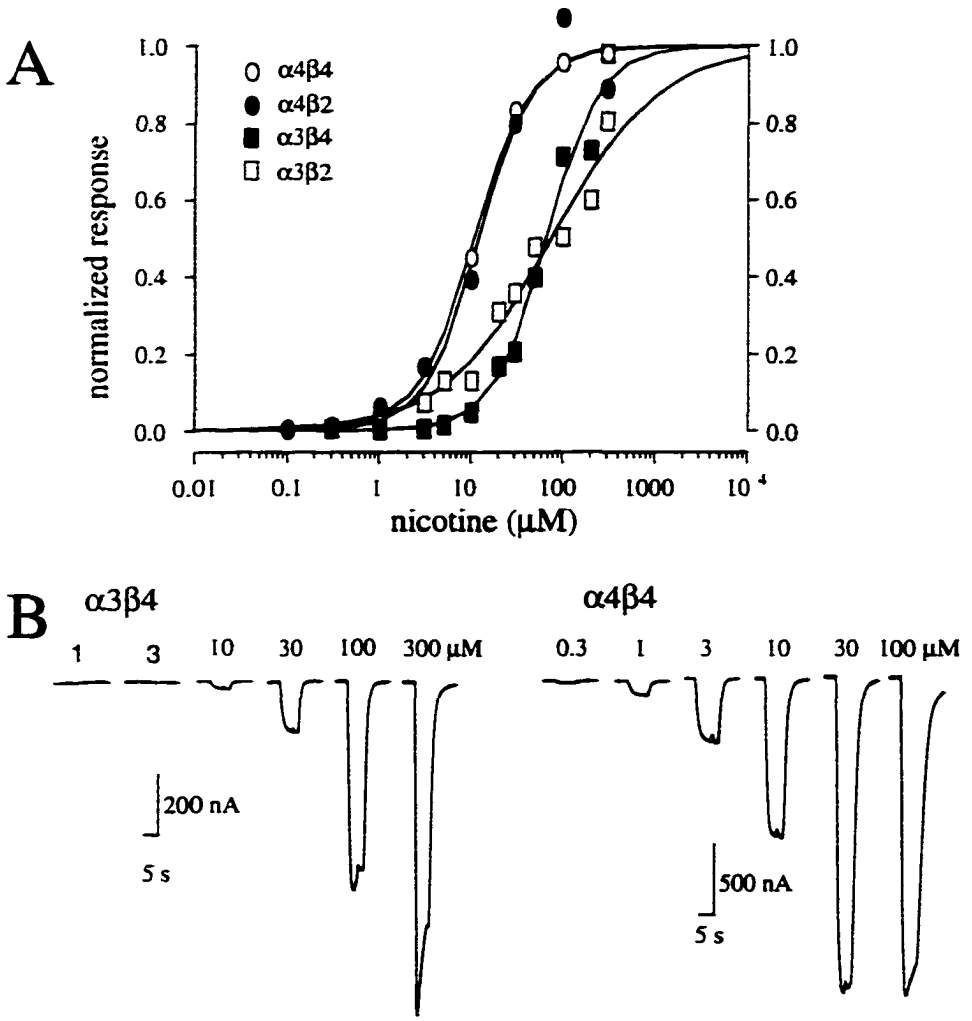


Figure 1. α4 subunits confer high apparent affinity on nAChRs. *A*, Concentration-response curves were constructed by measuring the peak current induced in response to brief (5-10 sec) applications of nicotine (100 nM-300 μM). The peak responses are plotted with respect to agonist concentration for four subtypes of nAChRs (α3β4, α3β2, α4β4, and α4β2). For each subtype, all responses were normalized to a response near the half-maximal concentration (EC_{50}). Each point represents one to eight separate measurements. The *solid lines* are logistic fits to the data. The calculated EC_{50} values and the Hill coefficients were, respectively, 65 μM and 1.5 (α3β4), 75 μM and 0.7 (α3β2), 11 μM and 1.4 (α4β4), and 12 μM and 1.5 (α4β2). *B*, Representative current responses for a series of nicotine concentrations applied to two different oocytes expressing either α3β4 receptors (*top*) or α4β4 receptors (*bottom*). All data shown in this figure were obtained in calcium-free conditions.

Table 2. Calcium-dependence of nAChR desensitization

Subtype	τ_{slow} (sec)		τ_{fast} (sec)		Fast component (%)		Fraction of cells with fast component	
	+Ca	-Ca	+Ca	- Ca	+Ca	-Ca[a]	+Ca	-Ca
α4β4	129.8±20.2	123.7 ±9.9	3.2 ±1.1	N/A	13.6 ±9.2	N/A	2/5	0/5
α4β2	90.7±5.9	102.5±20.0	5.4 ±1.5	7.4±1.4	40.3*±3.2	11.3±6.1	3/3	2/3
α3β4[b]	204.6±25.4	147.4±14.1	3.7*±0.7	11.3±0.9	21.8 ±3.7	18.5±3.5	6/11	2/11
α3β2	279.6±224	66.8 ±16.2	8.0 ±1.5	11.3±1.7	46.3*±8.6	66.8±1.0	4/4	4/4

All data were obtained from oocytes that were measured in both calcium-free (-Ca) and calcium-containing (+Ca) media.

[a] The mean percentage of fast component includes only those oocytes that had a measurable fast component.

[b] In some oocytes the two desensitization components were fitted separately (see Fig. 2).

* Indicates significance ($p < 0.05$), as compared with data obtained in calcium-free media.

apparent nicotine affinity. Because of the high permeability of various neuronal nAChRs to calcium (Mulle et al., 1992a; Vernino et al., 1992; Seguela et al., 1993; Rathouz and Berg, 1994) and the potential contamination of the measured currents with the activation of oocyte endogenous calcium-dependent Cl^- currents (Vernino et al., 1992), the above series of experiments was conducted in nominally calcium-free media. Physiologically relevant levels of calcium can, however, regulate nAChR activation; an effect that may result, in part, from changes in the affinity of nAChRs for agonist (Mulle et al., 1992b; Vernino et al., 1992; Galzi et al., 1996). To ascertain that the observed differences in the nAChR concentration-response relationships in calcium-free solutions were physiologically important, we repeated the experiments in calcium-containing media. The addition of calcium produced insignificant changes in EC_{50} values ($t = 0.35$; $p > 0.05$), and did not alter the order of potency of nicotine for the various nAChR subtypes (Table 1).

The much higher apparent nicotine affinity of α4-containing subtypes, as compared with α3-containing subtypes, is consistent with previous reports for heterologously expressed nAChRs from both chick (Hussy et al., 1994) and rat (Vibat et al., 1995). For α3 subunit-containing receptors, our estimated EC_{50} values for nicotine were comparable to previously published data (for review, see Role, 1992; McGehee and Role, 1995). Little or no comparable data exist for α4β4 receptors (Role, 1992). Our data for the α4β2 receptor predict a lower apparent nicotine affinity (15 μM, calcium-

Table1. Subunit-specific properties of nAChRs

Subtype	EC_{50}[a] (μM) +Ca	-Ca	IC_{50}[b] (μM) ±Ca	Desensitization at 500 nM (%) ±Ca	Desensitization time course[c] (τ_d; min) +Ca	-Ca	Desensitization recovery[d] (τ; min) ±Ca
α3β4	62	65	1.15	39	32.5 ±2.5 (3)	36.8 ±4.8 (7)	42.6 ±5.5 (4)
α4β4	9	11	0.042	87	35.5* ±3.2 (5)	5.2±2.8 (3)	413.8 [f]±305.5 (2)
α3β2	123	75	0.33	47	≈5 (3)[e]	3.9±2.1 (4)	11.7±4.1 (2)
α4β2	15	12	0.061	71	37±0.9 (4)	2.7±0.3 (2)	86.9 ±64.7 (4)
α7	90*	234	6.2 (-Ca) 1.3* (+Ca)	1 (-Ca) 12 (+Ca)	0.5±0.03(3)	1.9±0.5(4)	1.9 ±0.5(5)

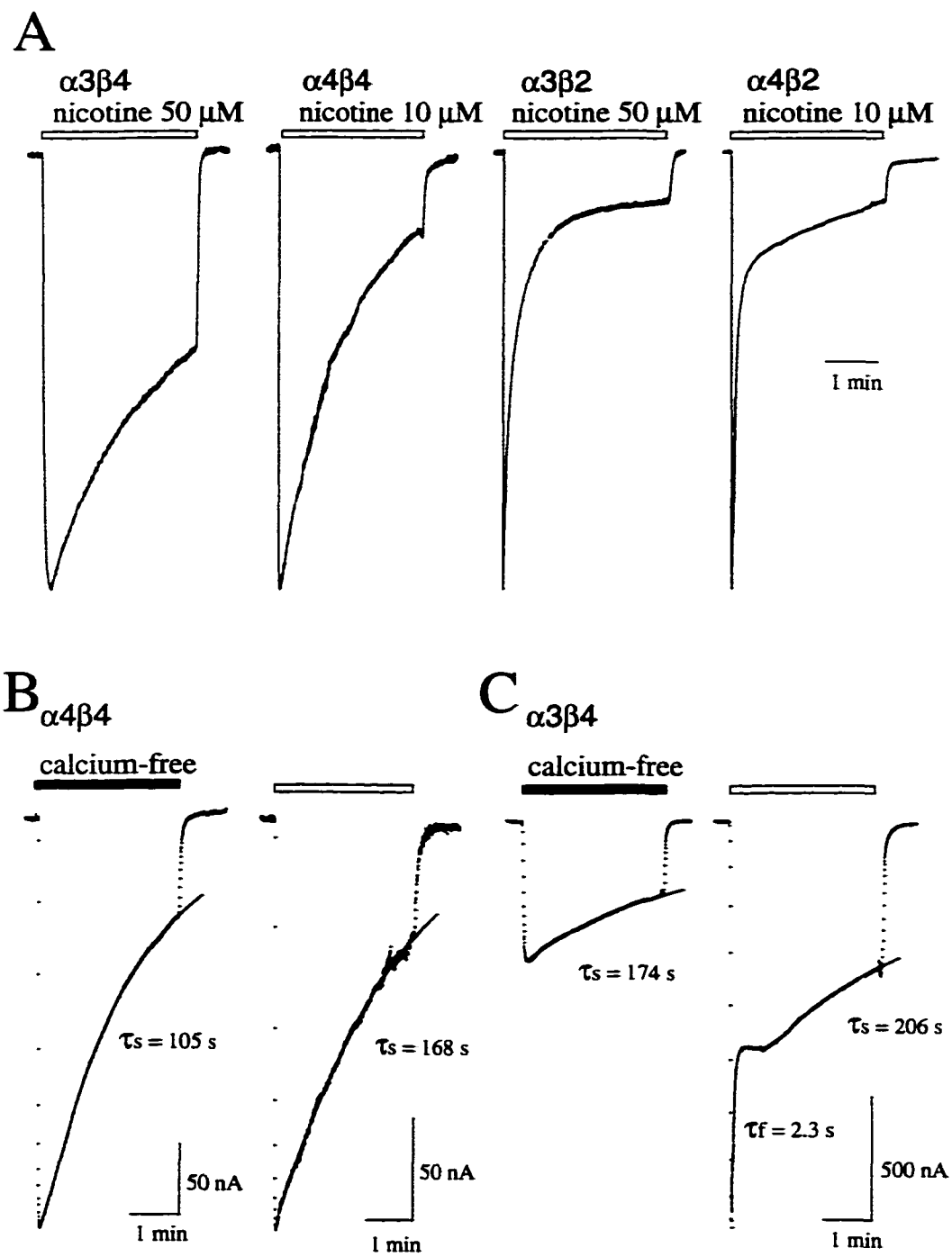
[a] EC_{50} calculated from the activation concentration-response curve in calcium-containing (+Ca) and calcium-free (-Ca) media.
[b] IC_{50} calculated from the concentration-response curves constructed from the mean desensitization produced in both +Ca and -Ca medium. These combined data are indicated by ± Ca.
[c] The time constant (τ_d) from a single exponential fit to the time course of desensitization at near IC_{50} concentrations of nicotine. These concentrations were 1 μM (α3β4); 100 nM (α4β4); 300 nM (α3β2); 300 nM (α4β2); 10 μM (-Ca; α7) and 3 μM (+Ca; α7). The number of measurements is indicated in parentheses.
[d] The time constant (τ) from a single exponential fit to the time course of recovery from desensitization at 1 μM nicotine for all subtypes except α7 (10 μM). The number of measurements is indicated in parentheses. In the majority of experiments, the exponential was fit assuming full recovery to control values.
[e] The reported values are estimates. It was difficult to obtain an accurate exponential fit for some of the α3β2 data sets, because the interpulse interval for the test pulses was large (5-10 min) and desensitization was mainly complete by the time of the first test pulse.
[f] Two additional oocytes expressing α4β4 receptors showed no recovery after 20-30 min wash.
* Indicates significance ($p < 0.05$) compared to data obtained in calcium-free media.

containing solutions) than reports of heterologously expressed α4β2 nAChRs from chick (0.8 μM; Bertrand et al., 1990), rat (0.3 μM; Vibat et al., 1995) and human (1.6 μM; Buisson et al., 1996).

Different Time Courses of Desensitization of nAChR Subtypes

Previous data show that nAChRs composed of different subunits desensitize with different time courses in response to various nicotinic agonists (Cachelin and Jaggi, 1991; Gross et al., 1991; 1995; Vibat et al., 1995). We have extended these findings, using nicotine as the agonist. Figure 2*A* shows representative responses of oocytes expressing four different nAChR subtypes to a 3 min application of nicotine at near half-maximal concentrations (see Fig. 1) in the presence of external calcium. Receptors containing β4 subunits desensitize slower than those containing β2 subunits ($t = 6.2$; $p < 0.05$). In most

Figure 2. The time course of desensitization varies with nAChR subtype. *A,* Responses to brief (≈ 3 min) applications of nicotine at near EC_{50} concentrations in calcium-containing media for oocytes injected with different subunit combinations. For comparison, the responses are normalized to their peaks. The time course of desensitization for responses obtained from oocytes expressing β4-containing receptors (*left two traces*) were well described by a single exponential component. For β2-containing receptors (*right two traces*), the decay phase was well described by the sum of two exponentials. *B,* Responses to 3 min applications of nicotine in calcium-free (*left trace*) and calcium-containing (*right trace*) media for an oocyte expressing α4β4 receptors. The traces are normalized to the peak currents. The solid lines are single exponential functions fitted to the desensitizing phase of the responses, and their associated time constants (τ_s) are shown. *C,* Responses to 3 min applications of nicotine in calcium-free (*left trace*) and calcium-containing (*right trace*) media for an oocyte expressing α3β4 receptors. The *solid lines* are single exponential functions fitted to the desensitizing phase of the responses. In calcium-containing media the data were fit with two separate single exponentials, designated τf and τs. The exponential functions contained a steady-state component, which was constrained not to fall below baseline current. The final steady-state values were generally in the range of 0-20% peak current.



experiments, the time course of desensitization for receptors containing α4 subunits was well described by a single exponential decay. In some oocytes, particularly those with larger peak currents, a faster "desensitizing" component was present in α4β4-expressing and α3β4-expressing oocytes (Fig. 2*C*). The fast component was calcium-dependent since it was largely eliminated when the same cells were examined in calcium-free media (Table 2). In contrast, the slower component of desensitization was unaffected by calcium (t = 0.21; $p > 0.05$; Fig. 2*B*; Table 2). These data are consistent with previous reports in which it was argued that a transient calcium-dependent Cl^- current could be activated by certain neuronal nAChRs (Vernino et al., 1992; Seguela et al., 1993). On the other hand, β2-containing receptors generally required the sum of two exponentials for a good fit to the data in both the presence and absence of calcium, although the relative amplitude of the fast component was significantly affected by calcium (t = 76.5; $p < 0.01$; Fig. 2; Table 2). Thus β2-containing nAChRs have, in addition to a slow component of desensitization, an intrinsic fast desensitization process. Differences in the size of the fast "desensitizing" component of α4β2 and α3β2 receptors in calcium-free and calcium-containing medium potentially could reflect differences in the extent of activation of the endogenous Cl^- current and/or differential modulation of the fast and slow components by external calcium ions.

Brief agonist applications do not provide sufficient data to fully characterize receptor desensitization (Katz and Thesleff, 1957; Feltz and Trautman, 1982). Moreover, the desensitization resulting from long applications of nanomolar concentrations of nicotine may reveal receptor subtype differences that are related more closely to mechanisms underlying nicotine dependency (Stolerman and Shoaib, 1991; Dani and Heineman, 1996). With this in mind, we examined the subunit dependence of nAChR desensitization induced by low levels of nicotine. Because it is not possible to measure desensitization directly from the very small currents produced by such low agonist concentrations, desensitization was estimated from the nicotine-induced reduction in the

current elicited by a brief (5-10 sec) test pulse of acetylcholine (ACh) near the half-maximal concentration for each receptor (Feltz and Trautman, 1982). The ACh test pulses were applied at sufficient time intervals (2-10 min, based upon the particular subunit combination) so as not to induce any additional desensitization. The data were expressed as the fractional test current remaining with respect to the time of nicotine exposure. For those experiments in which nicotine induced some receptor activation, the fractional current remaining was calculated from the sum of both the nicotine- and ACh-induced currents. Estimation of desensitization in this manner leads to an underestimation of the extent of desensitization if the current induced by nicotine is a significant fraction of the total response. Large nicotine-induced currents were apparent only at higher agonist concentrations with certain receptor subtypes (e.g., $\alpha3\beta4$; see Fig. 4). In these cases only, both the rate of desensitization development and the magnitude of desensitization will be slightly underestimated.

As shown in Figure 3, incubation of oocytes with nanomolar concentrations of nicotine (levels that produce little activation of any of the receptor subtypes; see Fig. 1) induces a profound desensitization of nAChRs. Differences in the time course of desensitization for different nAChR subtypes are immediately apparent and parallel the differences in desensitization observed with brief pulses (see Fig. 2). In our experiments, receptors with $\beta4$ subunits desensitized more slowly than those containing $\beta2$ subunits ($t = 9.7$; $p < 0.05$), irrespective of the presence or absence of calcium (Fig. 3). For all subunits the time course of desensitization could be described reasonably well by a single exponential fit (although see below), which allowed for a more quantitative comparison of desensitization kinetics. Table 1 shows that at concentrations of nicotine that produced a half-maximal block (see Fig. 5), the time constant for the development of desensitization varied considerably, from 4 min ($\alpha3\beta2$) to 45 min ($\alpha3\beta4$).

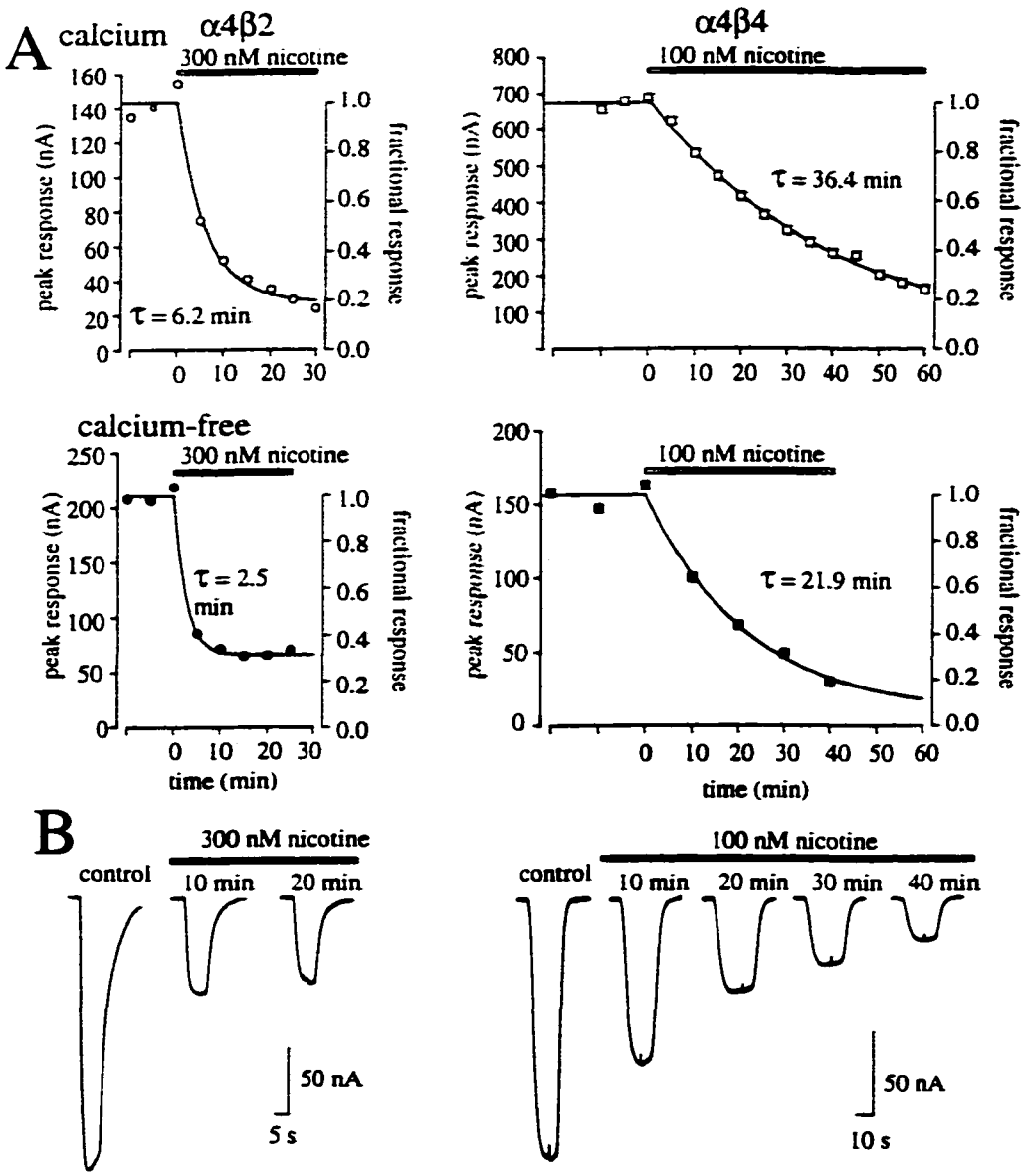


Figure 3. The time course of desensitization is slow in receptors containing β4 subunits. *A,* Plot of the peak current amplitudes induced by an ACh test pulse with respect to time before and during continuous perfusion with either 300 nM or 100 nM nicotine in oocytes expressing either α4β2 receptors (*left*) or α4β4 (*right*) receptors, respectively, in calcium-containing (*top traces*) or calcium-free (*bottom*) media. τ is the time constant for a single exponential fit to the desensitization time course. *B,* Representative traces of the ACh test pulses before (*control*) and at the indicated times following incubation with nicotine for individual α4β2- (*bottom left*) or α4β4- (*bottom right*) expressing oocytes in calcium-free media.

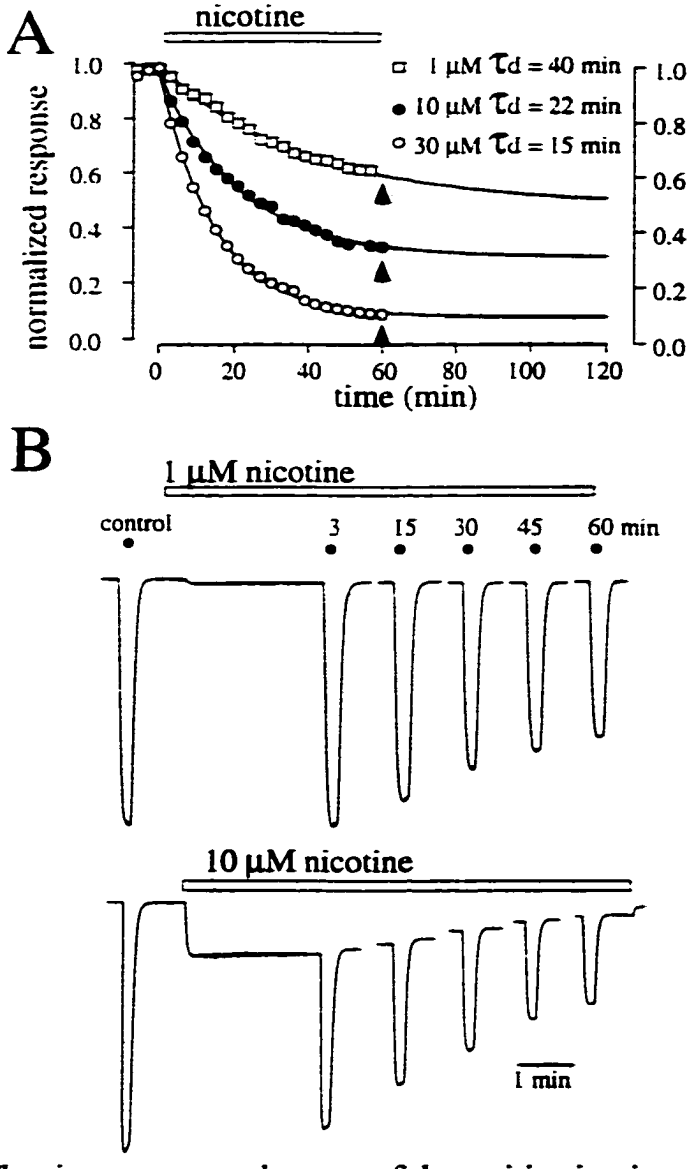


Figure 4. The time course and extent of desensitization is concentration -dependent. *A,* Plot of the peak amplitudes of the currents induced by ACh test pulses (100 μM) with respect to time before and during continuous perfusion with 1 μM (*open squares*), 10 μM (*filled circles*) or 30 μM (*open circles*) nicotine in oocytes expressing α3β4 receptors. τd is the time constant for a single exponential fit to the time course of desensitization. The *arrowheads* represent near equilibrium conditions at which the magnitude of desensitization was estimated (see Fig. 5). *B,* Representative traces of currents induced by ACh test pulses before (*control*) and at the indicated times during incubation with either 1 μM (*top traces*) or 10 μM (*bottom traces*) nicotine. All data were obtained in calcium-free media.

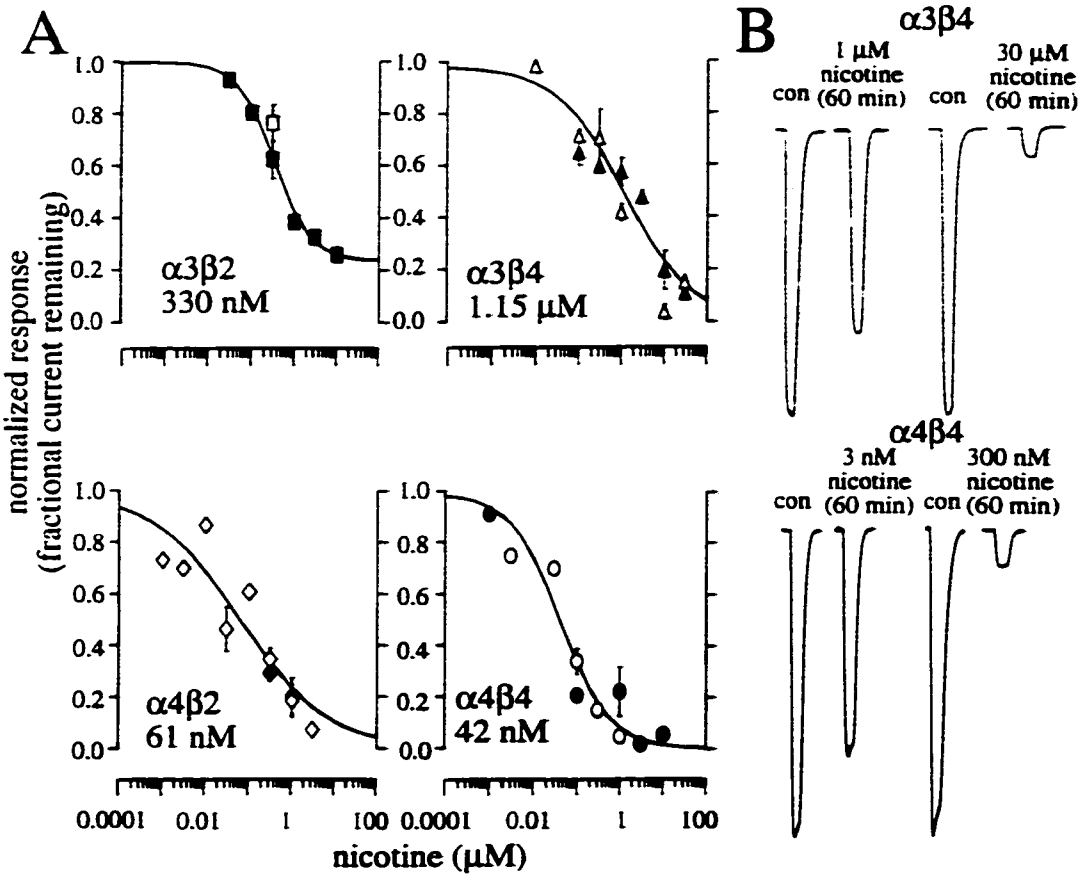


Figure 5. α4-containing nAChRs are desensitized by nanomolar concentrations of nicotine. *A*, Concentration-response plots of the ACh-induced fractional current remaining after chronic nicotine incubation in oocytes expressing α3β2, α3β4, α4β2, or α4β4 receptors. Some of the data were obtained by extrapolating the exponential fit to 60 min (α3β4 and α4β4) or 15-20 min (α3β2 and α4β2). The *open* and *filled* symbols represent data obtained in calcium-containing and calcium-free media, respectively. Each concentration *data point* represents between 1 and 10 measurements from separate oocytes. *Solid lines* are logistic fits to the mean of the all the data obtained in calcium-free and calcium-containing media. Fits were constrained so that the maximal block could not exceed 100% and so that at infinitely low nicotine concentrations the block was zero. The half-maximal concentration for inhibition (IC_{50}) by nicotine is shown for each nAChR subtype. *B*, Examples of the inhibition of the ACh test pulses by different concentrations of nicotine for two receptor subtypes, α3β4 (*top traces*) and α4β4 (*bottom traces*). Each pair of traces shows the current induced before nicotine incubation (*control*) and the current remaining after a 60 min incubation, with the concentration of nicotine indicated.

Potency of Nicotine for Desensitization of nAChRs

To compare more quantitatively the nicotine-induced desensitization of nAChR subtypes, we examined desensitization over a range of nicotine concentrations. Because the time course of desensitization development is concentration-dependent (Fig. 4) and subtype-dependent (see Fig. 3), different periods of nicotine incubation were required for each nAChR subtype to reach near-equilibrium conditions. Figure 4*A* shows that for the receptor with the slowest desensitization time course, α3β4, a 60 min exposure was sufficient to produce ~ 90 % desensitization even at low nicotine concentrations. Longer incubation times were typically not used to allow time for response recovery and to ensure that recovery reflected reversal of desensitization rather than *de novo* receptor synthesis (Peng et al., 1994; Hsu et al., 1995). In addition, for receptor subtypes that reached equilibrium faster (e.g. α3β2), nicotine exposure was continued in some cases for up to 30 min to ensure that a slower component of desensitization was not present. Although we cannot exclude completely the possibility that very slow desensitization components exist, desensitization of these subunits by nicotine is, for the most part, complete within the first hour (see also Hsu et al., 1995).

In theory, other mechanisms distinct from desensitization, such as partial antagonism or channel block by nicotine, could explain the decrease in ACh-induced currents in the presence of nicotine. Partial antagonism seems unlikely, especially for β4-containing receptors, because the block developed and recovered slowly. Even at low concentrations a purely competitive nicotine effect would be expected to be instantaneous, for the most part. Because nicotine is not a very efficacious agonist at chick α3β2 receptors (Wang et al., 1996) and given that this receptor subtype shows a relatively fast nicotine block, partial antagonism remains a possibility; however, nicotine does not appear to act as a partial antagonist at rat α3β2 nAChRs (Hussy et al., 1994). A direct, use-dependent channel block by nicotine, although reported for neuronal nicotinic

receptors, is also unlikely at such low agonist concentrations (Maconochie and Knight, 1992).

Once the time course of desensitization equilibrium was established, inhibition dose-response curves were constructed from the relative depression of an ACh test pulse induced during incubation with various nicotine concentrations (Fig. 5). The data were fit to a logistic equation from which the IC_{50} (half-maximal effective desensitizing concentration) for nicotine was estimated. In the present study we refer to the IC_{50} as the apparent affinity of nicotine for the desensitized state of the receptor. The receptor subtypes could be divided into two groups, based on these IC_{50} values. Similar to activation (see Fig. 1), nAChRs expressing α4 subunits had a higher apparent affinity for the desensitized state, as compared with nAChRs containing α3 subunits ($t = 5.6$; $p < 0.05$). There is an approximately linear relationship between the half-maximal concentrations of nicotine for activation and desensitization (Table 1). These data indicate that the time course of desensitization is not a good predictor of nicotine affinity for the desensitized state. For example, α4β4 receptors, which have a slow desensitization onset, demonstrate the highest apparent nicotine affinity for the desensitized state. On the other hand, α3β2 receptors, which have a fast onset, have a relatively low apparent desensitization affinity. At the highest concentrations of nicotine tested, all nAChR subtypes except α3β2 receptors were desensitized completely; α3β2 receptors were desensitized ~78 % (Fig. 5).

Recovery from Desensitization

After prolonged exposure to nicotine, the different nAChR subtypes recovered to pre-nicotine exposure values with different rates (Fig. 6). Differences in the time course of recovery from desensitization among subtypes were compared by fitting single exponentials to the recovery phase of the response after washout of 1 μM nicotine. Receptors containing α4 subunits (i.e., nAChRs with a higher-affinity desensitized state)

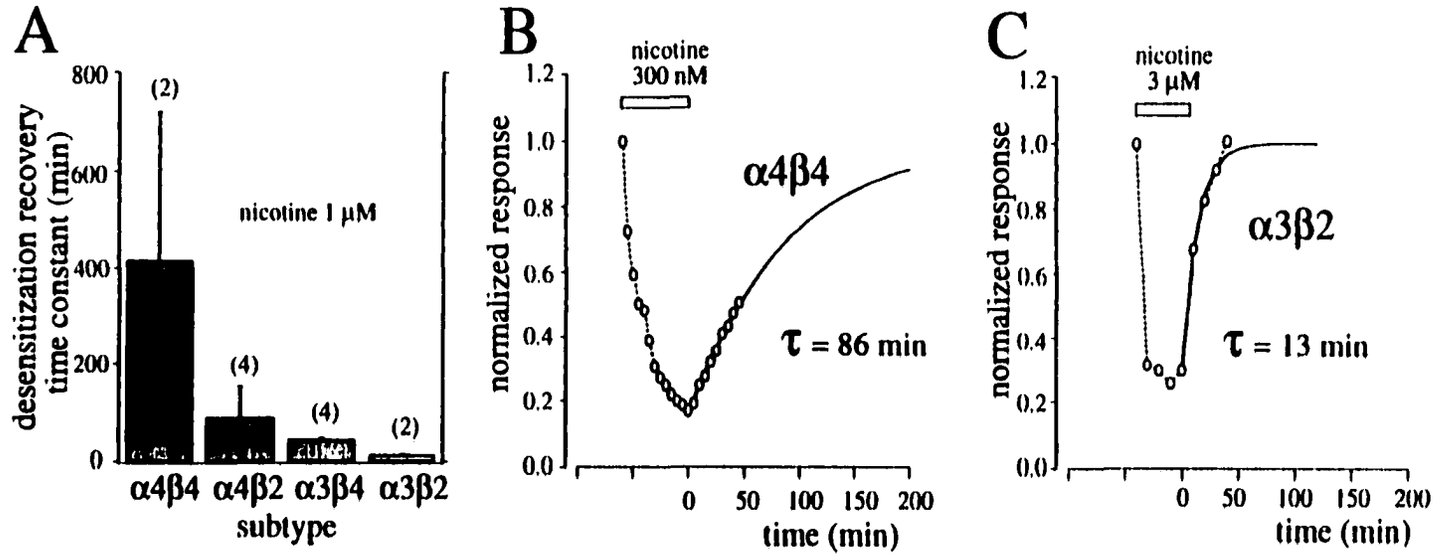


Figure 6. Time course of recovery from nicotine-induced desensitization is subunit-dependent. *A*, Histogram of the mean time constants for a single exponential fit to the time course of recovery from desensitization induced by 1 μM nicotine for a number of nAChR subtypes. Data from experiments in calcium-free and calcium-containing media were combined. The *number* of experiments for each subtype is indicated in *parentheses*. Recovery was estimated after nicotine incubation of 15-20 min (α3β2 and α4β2 receptors) and 60 min (α3β4 and α4β4 receptors). *B*, Plot of normalized peak current amplitudes (o---o) induced by a repetitively applied ACh test pulse (5 μM; 5 min intervals) with respect to time before, during (*open bar*) and after continuous perfusion with 300 nM nicotine in calcium-containing media for an oocyte expressing α4β4 receptors. *C*, Plot of normalized peak amplitudes of currents (o---o) induced by a repetitively applied ACh test pulse (100 μM; 10 min intervals) with respect to time before, during (*open bar*), and after continuous perfusion with 3 μM nicotine in calcium-free media for an oocyte expressing α3β2 receptors. The time constants (τ) in *B* and *C* result from single exponential fits (*solid line*) to the time course of recovery from desensitization. The exponential fit assumed recovery to control values.

recovered more slowly from desensitization than those containing α3 subunits ($t = 21.2$; $p < 0.01$). This result is not unexpected, because recovery ultimately involves an unbinding of agonist and a change in the receptor from desensitized to activatable; this is likely to be the slowest for the highest affinity receptor. Indeed, for α4β4 receptors, recovery was often incomplete even with 2 hr washout (Fig. 6*B*). On the other hand, α3β2 receptors recovered within a few minutes (Fig. 6*C*). Additionally, receptors containing β4 subunits (i.e., nAChRs in which the time course of desensitization development is slower) recovered more slowly than those containing β2 subunits ($t = 8.4$; $p < 0.05$; Fig. 6*A*). Thus, α3β4 receptors show a rate of recovery intermediate of α3β4 receptors and α4β4 receptors.

The above analysis assumed a single exponential recovery from desensitization. This is a reasonable assumption for β4-containing receptors; however, it may be inaccurate for β2-containing receptors. Receptors containing β2 subunits require the sum of two components to describe the desensitization phase during a brief pulse (see Fig. 2). These data imply multiple desensitized states for these receptors (Feltz and Trautman, 1982; Boyd, 1987). Thus, it follows that recovery from desensitization also may have two components (Feltz and Trautman, 1982; Cachelin and Colquhoun, 1989; Lester and Dani, 1995).

Recovery from desensitization for muscle-type nAChRs has been shown to be influenced by calcium-dependent mechanisms (Hardwick and Parsons, 1996). Thus, it is possible that similar mechanisms control the recovery of neuronal nAChRs. Because of the long duration of recordings necessary to examine the slow recovery from desensitization at low concentrations of nicotine, we used, instead, a paired-pulse approach to test the influence of calcium on the recovery from desensitization. A single 3 min application of nicotine at near EC_{50} concentrations was followed at a known interval by a second test pulse of nicotine, and the fractional recovery from desensitization was estimated in both calcium-free and calcium-containing media (Fig. 7). To reduce variability

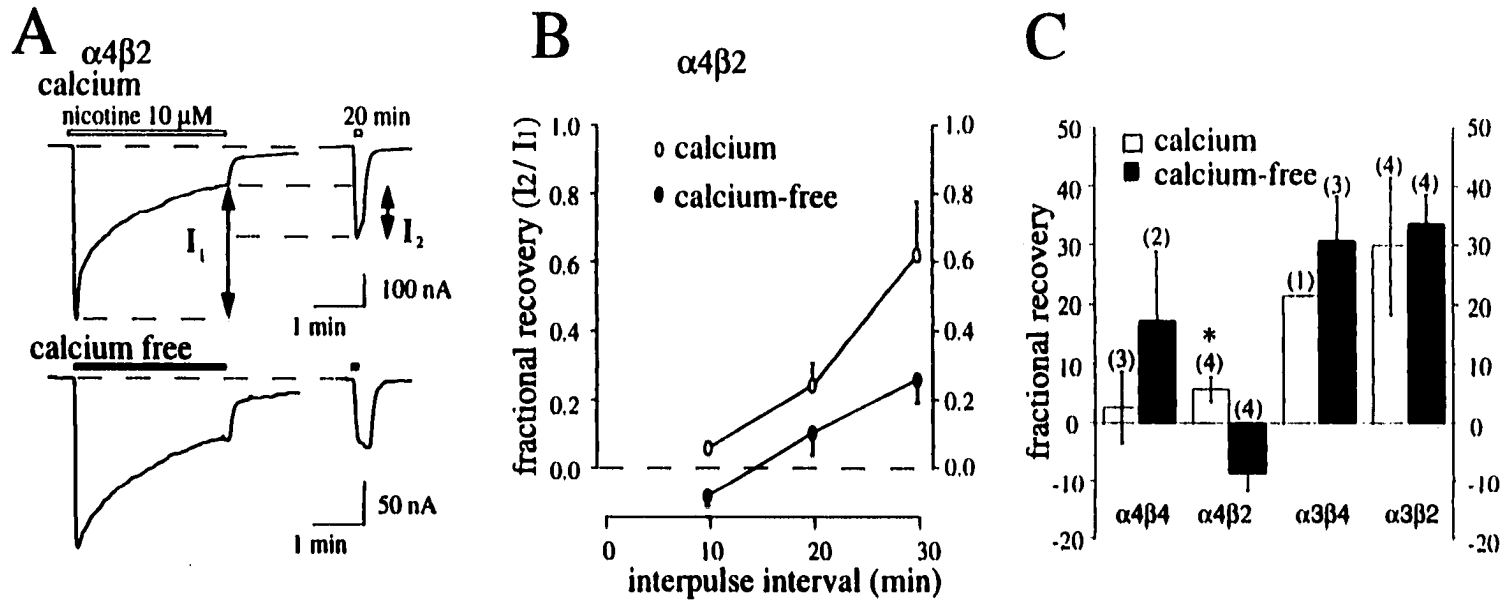


Figure 7. Subunit-specific differences in the recovery from desensitization. *A*, Paired-pulses of nicotine (10 μM) applied to an oocyte expressing α4β2 receptors at an interpulse interval of 20 min. To account for differences in the relative amount of desensitization in calcium-containing and calcium-free media, we quantified recovery by measuring the fractional increase in the amplitude of the second pulse (*I*2) with respect to the amount of desensitization (*I*1) induced by a 3 min application of nicotine. *B*, Plot of the fractional recovery from desensitization at various interpulse intervals in the presence (*open circles*) or absence (*filled circles*) of added calcium. *C*, Histogram of the relative recovery from desensitization for each nAChR subtype in the presence or absence of calcium. The *number* of experiments for each subtype is indicated in *parentheses*. The interpulse intervals were 10 min for both α4β4 receptors and α4β2 receptors, 5 min for α3β4 receptors, and 3 min for α3β2 receptors. Individual oocytes expressing α4β2 and α3β2 receptors were measured in both calcium-free and calcium-containing media. Significant differences ($P < .05$) between the results obtained in the two conditions are indicated by the asterisk.

often we tested the same oocyte under both conditions. Our results indicate that calcium has little effect on recovery from desensitization of nAChRs, although for some subtypes, e.g., $\alpha4\beta2$, the rate of recovery may be enhanced in the presence of calcium (t = 31.1; $p < 0.01$)

The outcome of prolonged exposure to nicotine is determined initially by the balance between receptor activation and desensitization at a particular nicotine concentration. To illustrate how this balance varies for different nAChR subtypes, we have replotted the activation and desensitization curves (Figs. 1*A*, 5) on the same axes (Fig. 8). The concentration range in which the activation and desensitization curves overlap indicates a "window current" (Steinbach, 1990), a region over which nicotine always produces some nAChR channel activation, because desensitization will be incomplete. At tobacco-related concentrations (60-300 nM; Benowitz et al., 1989) the main effect of nicotine will be to desensitize nAChRs (see Table 1), thus reducing the population of receptors available for potential stimulation by endogenously released ACh (see Lester and Dani, 1995). However, for the higher affinity $\alpha4$ subunit-containing nAChRs, nicotine at these concentrations will produce some channel activation.

$\alpha7$ Receptor Desensitization and Activation by Nicotine

Although the above data demonstrate that α and β subunits differentially influence the desensitization process, not all expressed nAChRs require β subunits for channel formation or, indeed, for desensitization (e.g., $\alpha7$ receptors; Couturier et al., 1990). For these receptors, desensitization characteristics must be determined solely by the α subunit (see Revah et al., 1991). The demonstration that $\alpha7$-containing receptors mediate a presynaptic release of transmitter in the continuous presence of low concentrations of nicotine (McGehee et al., 1995; Gray et al., 1996) demands a detailed investigation of this subtype with respect to desensitization.

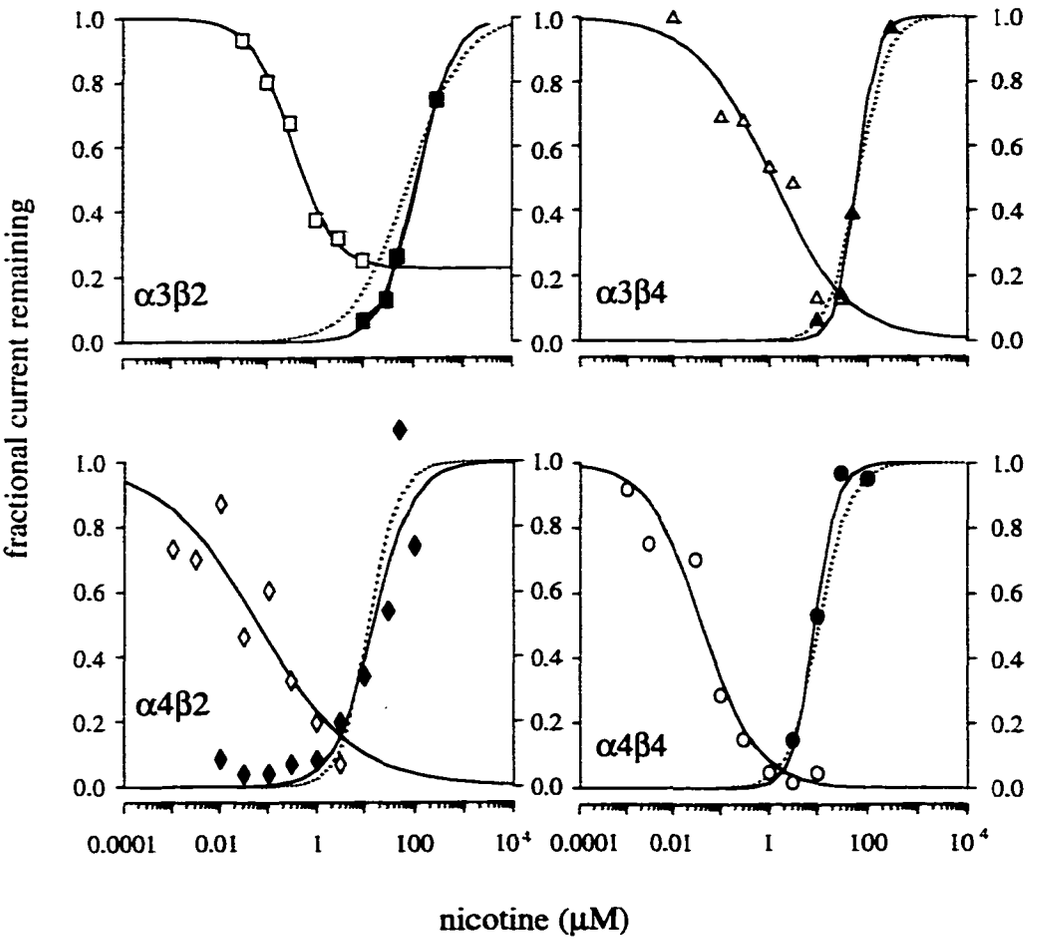


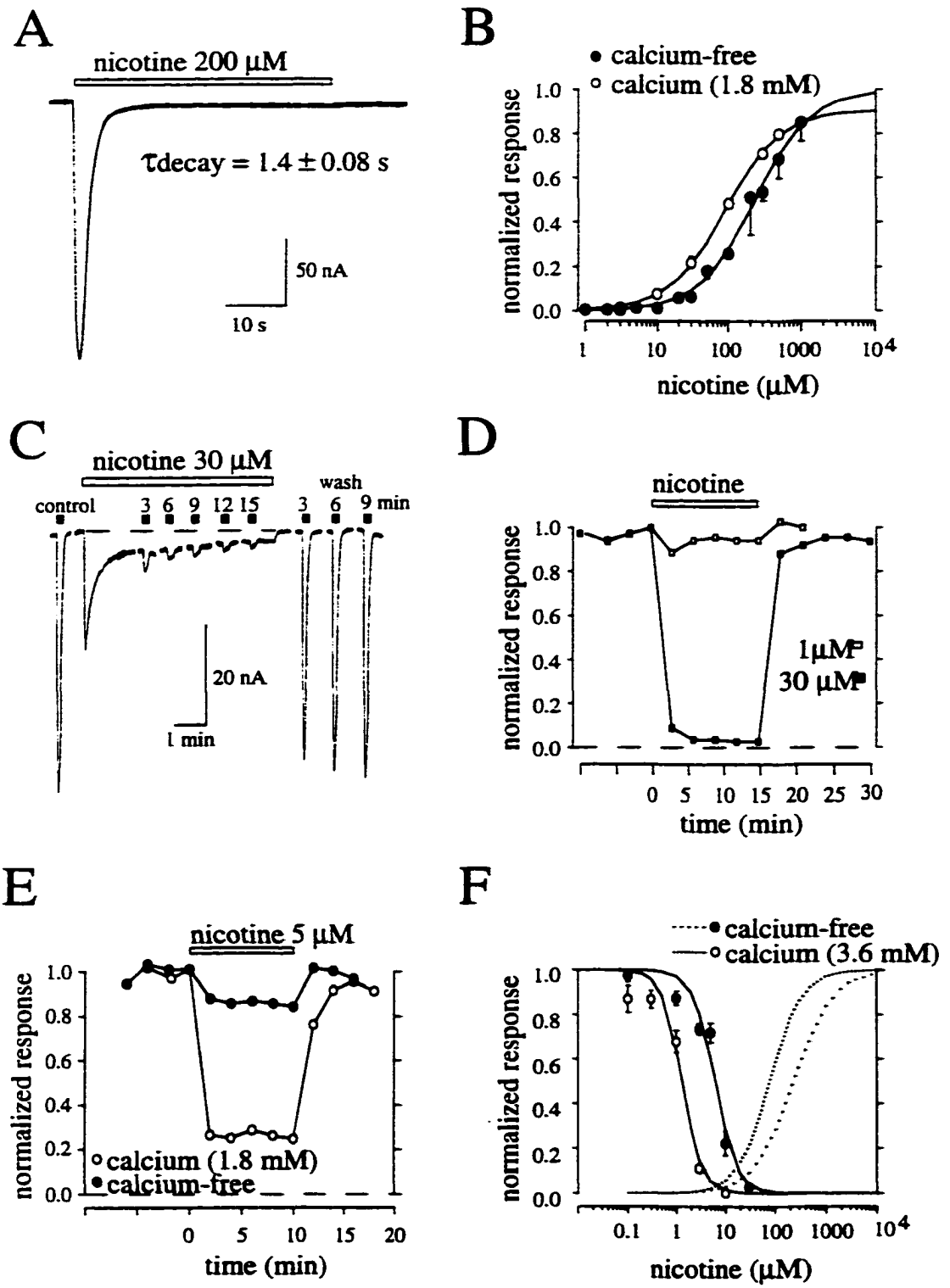
Figure 8. Receptor activation and desensitization have subtype-specific overlapping concentration ranges. Shown are summary concentration-response plots for activation in calcium-containing media (*filled symbols*; *solid lines*), and for desensitization (*open symbols*) of four nAChR subtypes. For comparison the logistic fits to the activation concentration-response curves obtained in calcium-free media are indicated with *dashed lines*.

Figure 9 characterizes activation and desensitization for α7 receptors. As described by others (e.g., Couturier et al., 1990; Revah et al., 1991; Seguela et al., 1993), α7 nAChRs activate and desensitize rapidly to brief applications of high agonist concentrations (Fig. 9*A*). The dose-response relationship for nicotine reveals a relatively high EC_{50} (234 μM, calcium-free solutions), implying that α7 receptors have a very low apparent affinity for nicotine. There was an approximately twofold shift in the EC_{50} to lower values in the presence of extracellular calcium ions ($t = 9.4$; $p < 0.05$) with little effect on maximal activation (Fig. 9*B*). Our EC_{50} values of 79 μM (3.6 mM calcium) and 90 μM (1.8 mM calcium) are slightly higher than those reported previously for human α7 (49 μM; Gopalakrishan et al., 1995), rat α7 (~30 μM; Seguela et al., 1993) and chick α7 (24 μM; Amar et al., 1993). Accounting for the decrease in single-channel conductance due to calcium (Mulle et al., 1992b; Amador and Dani, 1995), these data imply that calcium increases both the affinity and efficacy of α7 nAChRs (Galzi et al., 1995). Desensitization of α7 receptors (and subsequent recovery from desensitization) at low concentrations of nicotine was rapid (Fig. 9*C, D*). However, in the absence of added calcium, desensitization was observed only for concentrations of nicotine above approximately 1 μM, setting it apart from the various αβ-paired nAChRs. In calcium-free media, the half-maximal concentration of nicotine for desensitization was 6 μM, the highest of all receptors tested (Fig. 9*F*). The addition of calcium significantly increased the effectiveness of nicotine to desensitize α7-expressing receptors ($IC_{50} = 1$ μM; $t = 18.5$; $p < 0.01$), with little observable effect on the time course of desensitization ($t = 2.7$; $p > 0.05$; Fig. 9*E, F*). Thus, although α7 receptors exhibit pronounced and rapid desensitization, much higher concentrations of nicotine are required.

DISCUSSION

Examination of a number of expressed nAChRs reveals patterns of functional contributions of particular α and β subunits to the process of activation and of

Figure 9. Homomeric α7 receptors have low affinity for nicotine and rapid desensitization kinetics. *A,* Example of rapid desensitization induced by 200 μM nicotine in calcium-free media in an oocyte expressing α7 receptors. The mean time constant (τ_{decay}) resulting from a single exponential fit to the decay phase is indicated; the steady-state/peak current ratio was 0.023 ± 0.003 (10 observations from 4 cells). *B,* Concentration-response curves for nicotine-induced activation. The *solid lines* are logistic fits to the data sets. The EC_{50} values were 90 μM (n = 4) and 234 μM (n = 10), in the presence (*open circles*) or absence (*closed circles*) of calcium, respectively. The Hill coefficients for each were 1.1. *C,* Example of the currents induced by test pulses of ACh (100 μM) before (control), during continuous exposure, and after wash-out of 30 μM nicotine in calcium-free media. *D,* Plot of the peak test pulse amplitude (ACh, 100 μM) in two different oocytes expressing α7 receptors before, during, and after incubation with 1 μM (*open symbols*) or 30 μM (*closed symbols*) nicotine in calcium-free media. *E,* Plot of the peak test pulse amplitude induced by ACh before, during, and after incubation of an α7-expressing oocyte with a 10 min application of 5 μM nicotine in the presence (*open circles*) or absence (*closed circles*) of calcium. *F,* Concentration-response curve for nicotine-induced inhibition of the ACh test pulse in α7 receptor-expressing oocytes. The *solid lines* are logistic fits, and the half-maximal concentrations for inhibition (IC_{50}) by nicotine were 1.3 and 6.2 μM in the presence (*open circles*) or absence (*closed circles*) of calcium, respectively. The activation concentration-response curves obtained in the same conditions are shown (*dashed lines*) for comparison.



desensitization. For heteromeric nAChRs we find that the α subunit makes a significant contribution in determining the apparent nicotine affinity of the active and desensitized states of an nAChR: the β subunit makes a significant contribution in determining the overall time course of the development of desensitization of a nAChR. In addition, we have demonstrated that external calcium ions, while producing subtle effects on the kinetics and apparent affinities of nicotine for activation and desensitization of the αβ nAChRs, do not alter the pattern of contributions of these various subunits. In contrast, for the homomeric α7 nAChR, which displays faster kinetics and generally lower affinities for nicotine than the αβ pairs, we find that calcium increases the apparent affinity of nicotine for both the active and desensitized states.

Contribution of the α Subunit to Activation of nAChRs by Nicotine

Activation of various nAChR subtypes by nicotine produced straightforward results: α4-containing receptors have higher apparent affinities for nicotine than α3-containing receptors. Vibat et al. (1995) have observed a similar leftward shift in nicotine-induced dose-response curves on switching an α4 subunit for an α3 subunit in receptors containing rat β4 subunits. Chick and human α4β2 receptors (Bertrand et al., 1990; Peng et al., 1994; Buisson et al., 1996) are also activated more potently by nicotine than are α3β4 receptors (Hussy et al., 1994). If the α subunit is the principal agonist-binding subunit, these results could indicate differences in agonist-binding affinity between these two α subunits. However, this is likely an oversimplification. First, β subunits, as noted above, also affect the apparent affinity for some nAChR subtypes (Cachelin and Jaggi, 1991; Gross et al., 1991; Luetje and Patrick, 1991 Cohen et al., 1994), and, second, apparent affinity reflects both agonist binding and channel gating, so the observed subtype differences in concentration-response curves could represent differences in either affinity and/or efficacy. By examining the contribution of β subunits to the concentration-response relationship, Cohen et al. (1995) have argued that affinity differences cannot be

explained by a change in efficacy and that, by analogy with muscle nAChRs, the binding site is likely to form between both α and β subunits. This argument is supported by observations that antagonist sensitivity is also determined by both α and β subunits (Cachelin and Rust, 1995; Harvey and Luetje, 1996). It is possible, therefore, that our activation results are attributable to the fortuitous selection of particular nAChRs.

α and β Subunits Contribute to Nicotine- induced Desensitization

The desensitization time course for nAChR subtypes, measured either directly from the current decay during brief nicotine applications or from the decrease in response to ACh test pulses in the presence low concentrations of nicotine, is influenced for the most part by the β subunit; fast for β2-containing nAChRs and slow for β4-containing nAChRs. These data extend previous findings for rat nAChRs (Cachelin and Jaggi, 1991; Hsu et al., 1995). Because homomeric α7 receptors also desensitize, it is likely that the β subunit plays a modulatory, rather than a permissive role, in desensitization.

Studies investigating ACh desensitization of both chick and rat nAChRs, in which the β subunit was not varied, imply that the α subunit can also influence the time course of desensitization (Gross et al., 1991; Vibat et al., 1995). Aside from agonist-dependent differences in desensitization, the contribution of α subunits may be explained if one considers how desensitization was measured. For example, although short applications of nicotine demonstrate that α3β2 receptors desensitize faster than α4β2 receptors (Gross et al., 1991; Vibat et al., 1995), the reverse is true when the desensitization time course is followed by repetitive pulses (Vibat et al., 1995). Such differences may be explained if one considers a cyclical model for desensitization (Katz and Thesleff, 1957). In the continued presence of agonist, the time course of desensitization will reflect the rates governing equilibration between the active/open and the desensitized states. These rates would be expected to be faster for α3β2 receptors than α4β2 receptors; hence α3β2 receptors would desensitize faster in the continued presence of agonist. However, on

removal of agonist, recovery can occur by unbinding of agonist from the desensitized state (Dilger and Lui, 1993). Thus, if recovery in the absence of agonist is slow for α4β2 receptors as compared with α3β2 receptors, as we have observed, then desensitization measured by repetitive pulses (the agonist is absent in the interpulse interval) over a longer period of time would show a greater accumulation of α4β2 receptors in the desensitized state (Vibat et al., 1995).

Relationship Between nAChR Channel Properties and Receptor Regulation

Prolonged nicotine exposure leads to subtype-specific modulation (Schwartz and Kellar, 1985; Stolerman and Shoaib, 1991; Marks et al., 1993; Hsu et al., 1995; Dani and Heinemann, 1996). Because these changes arise at low nicotine concentrations, one must consider individual differences in nAChR behavior at these concentrations. A complete understanding of the cellular effects resulting from prolonged exposure to nicotine requires knowledge of the balance between activated and desensitized states of receptor subtypes (Steinbach, 1990; Balfour, 1994). All nAChRs studied desensitize at lower concentrations than they activate (Katz and Thesleff, 1957); however, for each receptor there is a concentration range over which desensitization is incomplete and activation has begun. Particularly for α4β2 receptors, this window approximately corresponds to the estimated levels of nicotine found in the brain after tobacco smoke inhalation (Benowitz et al., 1989).

For certain nAChR subtypes, e.g., α4β2 receptors, chronic nicotine exposure results in pronounced upregulation of receptor number (Flores et al., 1992; Peng et al., 1994; Gopalakrishnan et al., 1996). Because the desensitized state of α4β2 receptors has high nicotine affinity (it is desensitized for the most part by 1 μM nicotine), this state may be the trigger for the increase in receptor number (Ochoa et al., 1990; Peng et al., 1994; Gopalakrishnan et al., 1996). This idea is supported by evidence that exposure to nAChR antagonists also induces an increase in receptor number; i.e., a nonactive state of

the receptor is a sufficient stimulus (Collins et al., 1994; Peng et al., 1994; Gopalakrishnan et al., 1996). An accompanying increase in receptor sensitivity then could be a direct consequence of increased receptor number (Rowell and Wonnacott, 1990; Gopalakrishnan et al., 1996). However, others have observed a downregulation in receptor function (Marks et al., 1993; Collins et al., 1994; Peng et al., 1994). The relatively high activation affinity of $\alpha4\beta2$ receptors means that, although most receptors are desensitized by tobacco-related levels of nicotine, a continuous low level of activity remains. Prolonged low rates of synaptic activity have been associated with an NMDA-mediated calcium-dependent long-term depression of glutamatergic responses (Dudek and Bear, 1992; Mulkey and Malenka, 1992). An intriguing possibility is that functional downregulation of nAChRs is related to their high calcium permeability. The concept of an overlapping agonist concentration window for activation and desensitization could account for the dual up- and downregulation of $\alpha4\beta2$ receptor function (Peng et al., 1994; Gopalakrishnan et al., 1996); the precise agonist concentration would control the relative balance between active and desensitized receptor states.

Differences in regulation among nAChRs could be explained by subtype-specific nicotine sensitivities of both the desensitized and active receptor states. Studies on the homomeric $\alpha7$ receptor provide some support for this notion. The finding that nanomolar concentrations of nicotine can, via $\alpha7$-like presynaptic nAChRs, cause a continuous release of glutamate from synaptic terminals (McGehee et al., 1995; Gray et al., 1996) is consistent with the present observation that these concentrations of nicotine would cause little desensitization of $\alpha7$ receptors. The desensitization hypothesis for increases in nAChR number would predict that $\alpha7$ receptors should not be upregulated as readily as $\alpha4\beta2$ receptors. In fact, the number of α-bungarotoxin (α-BTX) binding sites, putatively formed from $\alpha7$ subunits (Couturier et al., 1990), can be upregulated by chronic nicotine treatment. However, relatively high concentrations of nicotine are required (Marks et al.,

1983). At lower concentrations of nicotine (that result in increased $\alpha4\beta2$ receptor number), $\alpha7$/α-BTX receptors are unaffected (Marks et al., 1985; Collins et al., 1994).

It should be noted that some CNS nAChRs, including $\alpha7$, may contain more than a single type of α or β subunit (Conroy et al., 1992; Anand et al., 1993; Ramirez-Latorre et al., 1996; Wang et al., 1996). Also, there may be some differences between nAChRs expressed in oocytes and mammalian cells (Peng et al., 1994; Wong et al., 1995; Buisson et al., 1996). Such differences may result in shifts in concentration-response curves and/or desensitization properties (Ramirez-Latorre et al., 1996; Wang et al., 1996). Regardless, knowledge of the subunit contribution to activation and desensitization is important for accurate predictions of the differential effects of tobacco-related levels of nicotine on CNS nAChRs.

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UPREGULATION OF SURFACE α4β2 NICOTINIC RECEPTORS IS INITIATED BY RECEPTOR DESENSITIZATION AFTER CHRONIC EXPOSURE TO NICOTINE

by

CATHERINE P. FENSTER, TERRI WHITWORTH, MICHAEL W. QUICK, AND ROBIN A.J. LESTER

The Journal of Neuroscience (1999) 12:4804-4814

ABSTRACT

It is hypothesized that desensitization of neuronal nicotinic acetylcholine receptors (nAChRs) induced by chronic exposure to nicotine initiates upregulation of nAChR number. To test this hypothesis directly, oocytes expressing α4β2 receptors were chronically incubated (24-48 hr) in nicotine, and the resulting changes in specific [^{3}H]nicotine binding to surface receptors on intact oocytes were compared with functional receptor desensitization. Four lines of evidence strongly support the hypothesis: (1) The half-maximal nicotine concentration necessary to produce desensitization (9.7 nM) was the same as that needed to induce upregulation (9.9 nM). (2) The concentration of [^{3}H]nicotine for half-maximal binding to surface nAChRs on intact oocytes was also similar (11.1 nM), as predicted from cyclical desensitization models. (3) Functional desensitization of α3β4 receptors required 10-fold higher nicotine concentrations, and this was mirrored by a 10-fold shift in concentrations necessary for upregulation. (4) Mutant α4β2 receptors that do not recover fully from desensitization, but not wild-type channels, were upregulated after acute (1 hr) applications of nicotine. Interestingly, the nicotine concentration required for half-maximal binding of α4β2 receptors in total cell membrane homogenates was 20-fold lower than that measured for surface nAChRs in intact oocytes. These data suggest that cell homogenate binding assays may not accurately reflect the *in vivo* desensitization affinity of surface nAChRs and may account for some of the previously reported differences in the efficacy of nicotine for inducing nAChR desensitization and upregulation.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels activated by the endogenous neurotransmitter acetylcholine and exogenous drugs, such as nicotine (Role, 1992; Sargent, 1993). The effect of both acute and chronic nicotine on the activity of different nAChR subtypes may be relevant to tolerance,

dependence, and withdrawal symptoms associated with nicotine addiction (Wonnacott, 1990; Balfour, 1994; Dani and Heinemann, 1996). One result of chronic exposure to tobacco-related levels of nicotine (Benowitz et al., 1989) is the upregulation of high-affinity $\alpha4\beta2$ subunit-containing [^{3}H]nicotine binding sites in the CNS (Marks et al., 1983; Benwell et al., 1988; Flores et al., 1992, 1997; Breese et al., 1997). Such upregulation of receptor number may contribute to the addiction process. The mechanism by which chronic nicotine exposure leads to receptor upregulation is not known.

In addition to upregulation of receptor number, chronic nicotine exposure can be associated with a long-lasting downregulation in receptor responsiveness (Lukas et al., 1991; Marks et al., 1993; Peng et al., 1994; Hsu et al., 1996). This decrease in function is thought to be, at least in part, a consequence of agonist-induced desensitization (see Boyd, 1987). In addition, nicotine-induced receptor desensitization has been hypothesized to be responsible for receptor upregulation (Marks et al., 1983; Schwartz and Kellar, 1985). A straightforward prediction of this hypothesis is that nicotine concentrations that produce receptor desensitization should also induce receptor upregulation. Results of several studies suggest this prediction to be false, because nicotine concentrations necessary to induce desensitization and upregulation can differ by several orders of magnitude (Peng et al., 1994; Bencherif et al., 1995; Whiteaker et al., 1998). However, these conclusions are based on several assumptions. First, that agonist equilibrium binding assays accurately assess the desensitized state(s) of the receptor. This may not be true. Agonist dose-dependencies for equilibrium binding and functional estimates of desensitization can differ by several orders of magnitude (Marks et al., 1996); such differences would account for the apparent nicotine concentration mismatch between measures of desensitization and upregulation. Second, agonist binding measured in standard membrane homogenization assays are assumed to reflect agonist binding to

functional, intact receptors, although some evidence suggests that this is not true (Whiteaker et al., 1998).

A more direct test of the prediction would be to assess desensitization functionally and compare this result with receptor upregulation measured by agonist binding to intact, cell-surface receptors. In the present study, $\alpha4\beta2$ nAChRs expressed in *Xenopus* oocytes were chronically incubated in nicotine. Nicotine dose-response relationships were constructed for functional receptor desensitization and for receptor upregulation assessed by surface [^{3}H]nicotine binding to intact oocytes. To further examine the hypothesis that nicotine-induced receptor desensitization is responsible for receptor upregulation, we also tested the predictions that nAChRs with lower affinities for functional desensitization will also have a lower affinity for receptor upregulation, and that mutant $\alpha4\beta2$ nAChRs that fail to recover fully from desensitization will upregulate following the removal of nicotine.

MATERIALS AND METHODS

Xenopus *Oocyte Preparation and cRNA Injection*

Procedures for preparation of oocytes have been described in detail previously (Quick and Lester, 1994). Briefly, oocytes were defolliculated and maintained at 18° C in incubation medium containing ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4), 1.8 mM $CaCl_2$, 50 μg/ml gentamycin, and 5% horse serum. Subunit cRNAs were synthesized *in vitro* (Message Machine; Ambion, Austin, TX) from linearized plasmid templates of rat cDNA clones. A mutant α4 subunit ($\alpha4^{S336A}$) was created in which a PKC consensus serine phosphorylation site was mutated to alanine (pALTER 1; Promega, Madison, WI). The mutation was verified by sequencing (Fenster et al., 1999). Oocytes were injected with 25 ng of cRNA/subunit/oocyte; α and β subunit cRNAs were injected in 1:1 ratios. All salts and drugs were obtained from Sigma (St. Louis, MO).

Electrophysiology

Whole-cell currents were measured at room temperature (20-25^{0}C), 24-120 hr postinjection, with a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA) in a standard two-microelectrode voltage-clamp configuration. Electrodes were filled with 3 M KCl and had resistances of 0.5-2 MΩ. Oocytes were clamped between -40 mV and -60 mV and superfused continuously in ND96 containing 1.8 mM $CaCl_2$ (ND96+Ca). Nicotine was applied in these solutions. (-)-Nicotine tartrate (nicotine) was prepared from frozen stock solutions. All currents were recorded on a chart recorder and/or on an 80486-based computer with AxoScope software (Axon Instruments) after 50-100 Hz low-pass filtering at a digitization frequency of 200 Hz. Solutions were gravity-fed via a six-way manual valve (Rainin Instruments, Woburn, MA) to the oocyte in the recording chamber. Solution exchange considerations are discussed by Fenster et al. (1997).

Criteria for Functional Data Selection

Except for the experiments shown in Figure 1*B*, oocytes with initial nicotinic response amplitudes >3 μA and <50 nA were not included in the data analysis. Additionally, responses were required to be at least twofold greater than the holding current, and the holding current at a given membrane potential was required to be <100 nA. Functional receptor desensitization is usually calculated as the reduction in response amplitude induced by a brief test pulse of agonist after a period of continuous incubation with the desensitizing agent (Katz and Thesleff, 1957: Feltz and Trautman, 1982). However, because of the long incubations (24–48 hr) in the present study, it is necessary to correct this measurement for any time-dependent changes in basal receptor expression-function that occur independent of nicotine exposure. Thus, response amplitudes in control oocytes from the same batch (not incubated in nicotine) were monitored over the same period. For each oocyte incubated in nicotine (nic), the fractional desensitization was calculated as ratio of the current amplitude (I) at time (t) to

the initial current amplitude at $t = 0$, $\Delta_{nic} = I_{nic(t)} / I_{nic(t = 0)}$. This value was then normalized for changes in control receptor function, $\Delta_{con} = I_{con(t)} / I_{con(t = 0)}$, obtained from control (con) oocytes (see Fig. 3). Thus, the overall estimate of desensitization was calculated as $\Delta_{nic} / \Delta_{con}$ (see Fig. 3). For statistical comparison of mean data, weighted means *t* tests were performed.

[^{3}H]nicotine Binding Assays

Binding assays were performed on both intact oocytes and on oocyte membranes after homogenization. Binding to intact oocytes was performed essentially as described previously (Chang and Weiss, 1999). Uninjected oocytes or nAChR-injected oocytes were visually inspected to ensure that collagenase treatment (2 mg/ml; Type A; Boehringer, Indianapolis, IN) successfully removed follicle cells from around the oocyte membrane. The presence of the follicle layer resulted in high, nonspecific [^{3}H]nicotine labeling that prevented accurate assessment of specific [^{3}H]nicotine binding (unpublished observations). The oocytes were removed from incubation media and rinsed in ND96+Ca for several minutes. Individual oocytes were next placed in a single well of a 96-well plate containing 40 µl of ND96+Ca. Stocks (5X) of [^{3}H]nicotine [((-)-[N-methyl-^{3}H]nicotine, 82.0 Ci/mmol; DuPont-NEN) were prepared by dilution in ND96+Ca. The assay (60 min at room temperature) was initiated with the addition of 10 µl of 5X [^{3}H]nicotine to the well, followed by gentle trituration of the media for several seconds so as not to disrupt the integrity of the cell. The assay was terminated in one of two ways: The oocyte was either suspended from the cut end of a pipette tip by light suction or pipetted into the cut end of a pipette tip along with 4 µl of assay solution. In the former case, the oocyte was then submerged sequentially into four different 2.5 ml wells containing ice-cold ND96+Ca. The total wash time for all four wells was 16 sec (4 sec/well). In the latter case, the oocyte was dropped into each wash well and repipetted into the next well. Based on a set of control experiments, the total wash time for all four

wells was 18.8 ± 2.1 sec (n = 15 trials). Radioactivity was measured in one of two ways. After the final wash, the oocyte was placed directly into a scintillation vial containing nonaqueous liquid scintillation cocktail (Scintisafe F; Fisher Scientific, Houston, TX). The amount of radioactivity was estimated within 2 min of the wash step. In some experiments after washing, the oocyte was placed in one well of a 96-well plate containing ND96+Ca, and the bound [^{3}H]nicotine was allowed to dissociate (see below, Fig. 2*C*). The contents of each well were then subjected to liquid scintillation counting. Where compared, these two methods yielded similar results. Oocyte batches in which nonspecific, [^{3}H]nicotine binding to uninjected oocytes was higher than 100 cpm were not used in data collection; based on this constraint, ~30% of the oocyte batches were usable.

Specific [^{3}H]nicotine Binding and Channel Function

Direct comparison of functional desensitization and receptor upregulation in intact oocytes has three requirements: that we can accurately measure specific [^{3}H]nicotine binding; that the measures of specific binding correlate with functional expression; and that the measured binding is occurring only on surface nAChRs. Control experiments demonstrating measurement of specific [^{3}H]nicotine binding are shown in Figure 1*A*. Oocytes expressing α4β2 nAChRs were incubated for 1 hr in various concentrations of [^{3}H]nicotine. Specific binding was calculated as the total cpm minus the nonspecific cpm; nonspecific cpm was defined as the cpm of oocytes measured in the presence of 100 μM unlabeled acetylcholine. These data show that [^{3}H]nicotine binding to intact oocytes is saturable and that nicotinic receptor agonists are competitive with [^{3}H]nicotine binding. Figure 1*B* shows that α4β2 nAChRs currents activated by saturating concentrations of nicotine (100 μM) and subsequent [^{3}H]nicotine binding (60 nM) to the same intact oocyte are highly correlated (r = 0.94).

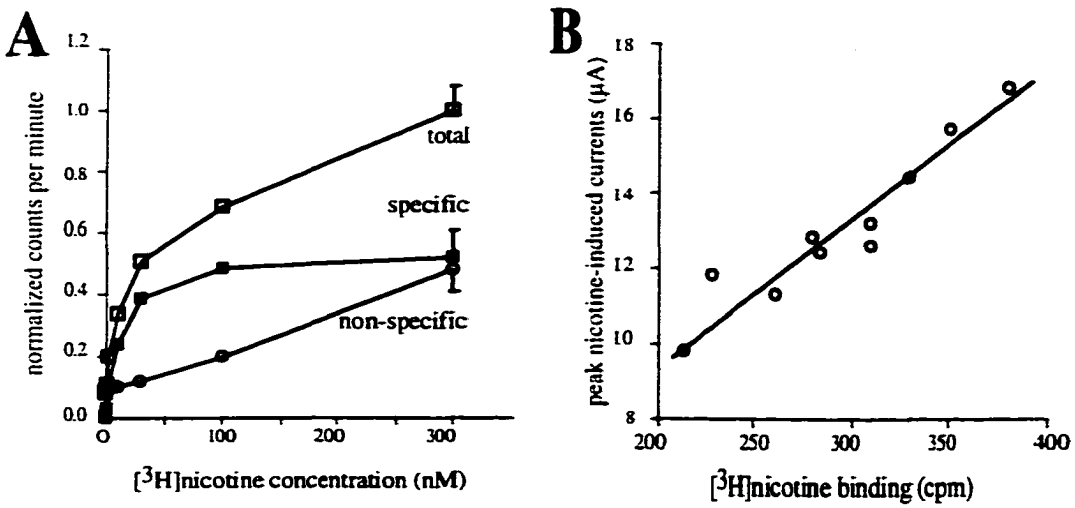


Figure 1. Specific [^{3}H]nicotine binding to intact oocytes. Oocytes were injected with α4 and β2 subunit cRNAs and assayed 3 days after injection. *A*, [^{3}H]Nicotine binding is saturable. Individual α4β2-expressing oocytes were incubated in various [^{3}H]nicotine concentrations (0. 0.3, 1, 3, 10, 30, 100 and 300 nM) for 60 min. [^{3}H]Nicotine binding was determined in the absence (total [^{3}H]nicotine binding, *open squares*) or presence (nonspecific [^{3}H]nicotine binding, *open circles*) of 100 μM nonradiolabeled acetylcholine. Specific [^{3}H]nicotine bidning (*filled squares*) was determined by subtracting nonspecific counts from total counts. Each data point represents the measurement of five to six oocytes; for clarity, the mean ± SEM across all nicotine concentrations is plotted with the symbols at the 300 nM nicotine value. *B*, Specific [^{3}H]nicotine binding is correlated with nicotine-induced currents. α4β2-expressing oocytes were voltage-clamped at -40 mV, and peak currents were elicited using 100 μM nictotine. Specific surface [^{3}H]nicotine binding was then determined in these same oocytes by incubation in 60 nM [^{3}H]nicotine for 1 hr. The correlation for these two measurements was 0.94.

From the slope of the regression fit (37 nA/cpm), it is possible to calculate the number of bound nicotine molecules per functional channel. The number of functional channels can be estimated from the ratio of the measured peak current to the single channel current, assuming that all the channels are open at the peak. The single channel current will be 0.8 pA at –60 mV using a single channel conductance of 13.3 pS (Papke et al., 1989). Thus there will be $\approx 4.625 * 10^4$ nAChR channels/cpm. The number of bound nicotine molecules per cpm was estimated as $6.12 * 10^6$ from the following:

$$\text{molecules} = \frac{N_A}{3.7 * 10^{10} . S_A . f} \qquad (1)$$

where S_A is the specific activity of [^{3}H]nicotine in Curie per moles, f is the factor for converting disintegration per second to counts per minute with a counting efficiency of 0.54, and N_A is Avagadro's number. Thus, these data predict 132 molecules of nicotine for each functional channel. Assuming that two molecules of nicotine bind per channel, these data imply that only ~2% channels are functional. In terms of a single oocyte with ~ 300 cpm (see Fig. 1*B*), the number of bound [^{3}H]nicotine molecules would be $\sim 1.8 * 10^9$, corresponding to $\sim 10^9$ total receptors, of which $\sim 2 * 10^7$ would be functional. Because of some nonspecific binding in this instance, and a likely underestimation of the number of channels as a result of noninstantaneous solution-exchange, this will be a lower limit for the percentage of functional channels. With ~20 % nonspecific binding (see Fig. 2*A*) and assuming only one-third of channels are open at peak, the number of functional channels could be ~6%. Similar discrepencies between toxin binding and channel conductance have been noted for sodium channels (Ritchie and Rogart, 1977). For nAChRs reported here, the excess silent receptors could represent a reserve pool and/or desensitized receptors (Margiotta et al., 1987; Bencherif et al., 1995). Because $\alpha 4\beta 2$ nAChRs can enter persistently inactive conformations after chronic nicotine treatment (Lukas, 1991; Peng et al., 1994), it is not unreasonable to suggest that such

conformations may pre-exist on the cell surface. Because of the strong correlation between the amounts of total binding and currents (Fig. 1*B*), functional and silent receptor pools are likely to be in equilibrium. Thus, despite the apparent excess of binding sites, these data strongly support the suggestion that functional nAChRs are a subpopulation of the receptor pool that is measured in a binding assay.

[^{3}H]nicotine Labels Surface Receptors in Intact Oocytes

Three sets of experiments were performed in order to verify that the binding assays were measuring specific binding to surface nAChRs (i.e., to eliminate the contribution of radioactivity associated with internalized [^{3}H]nicotine). Two experiments were designed to test that the nonaqueous scintillation cocktail was only counting external [^{3}H]nicotine. (1) Nonexpressing oocytes were injected with 5000 cpm (as determined by prior scintillation counting of various [^{3}H]nicotine aliquots). Intact oocytes were then subjected to liquid scintillation counting. Some oocytes were then crushed and recounted; others were removed from the nonaqueous scintillation cocktail and placed in aqueous scintillation cocktail (Scintisafe Econo 1; Fisher Scientific). The crushed oocytes and the intact oocytes counted in aqueous scintillation cocktail revealed radioactive emissions of ~5000 cpm; the intact oocytes measured in nonaqueous scintillation cocktail revealed radioactive emissions of approximately 20 cpm (0.004% of total injected counts, $n = 6$). (2) [^{3}H]nicotine binding assays were performed on oocytes expressing $\alpha4\beta2$ nAChRs using 60 nM [^{3}H]nicotine. Specific binding was determined as described above (Fig. 1*A*). One subset of oocytes ($n = 5$) was counted by placing the intact oocyte in nonaqueous scintillation cocktail; the counts from a second subset of oocytes from the same oocyte batch were determined by permitting the radioactivity to dissociate for 2 min into a well containing ND96+Ca and counting the well contents. If a significant amount of [^{3}H]nicotine was internalized and subsequently counted, then the counts from the intact oocyte should be much higher than in the dissociation study.

However, adjusting for the rate of dissociation (see below; Fig. 2*C*), the number of counts measured in the intact oocytes (167 ± 23 cpm; $n = 7$) was comparable to that counted by the dissociation method (138 ± 30 cpm; $n = 4$).

Although these experiments suggested that internal radioactivity will not be counted, a potential confound is that any nonspecific internal accumulation of [^{3}H]nicotine during a 1 hr incubation would begin to leak back out of the oocyte between the end of the assay and the scintillation counting step (~2 min). To examine the contribution of this effect to our measurements, we put a known number of counts of [^{3}H]nicotine (corresponding to 300 nM [^{3}H]nicotine, the highest concentration we used in the experiments described in this paper) into one well of a 96-well plate with one nonexpressing oocyte and determined the amount of [^{3}H]nicotine that accumulated inside of the oocyte during 1 hr. This amount was between 6 and 11% of the starting external value and varied based on the oocyte batch (three oocyte batches, seven oocytes per batch). We then injected other nonexpressing oocytes with [^{3}H]nicotine corresponding the number of counts internalized in 1 hr and determined the number of counts that emerged from these oocytes in 2 min. At the highest concentrations tested, this amount corresponded to 9% of the injected counts. Thus, the maximum contamination resulting from the re-emergence of internalized counts during an assay would be ~1%.

These controls demonstrate that, although [^{3}H]nicotine will accumulate inside the oocyte during a 1 hr assay, it will not be detected by counting intact oocytes in nonaqueous scintillation cocktail. Any [^{3}H]nicotine that leaks back out will contribute negligibly to the total number of counts. Thus, ACh-displacable [^{3}H]nicotine binding to intact oocytes will mainly reflect surface membrane nAChR labeling.

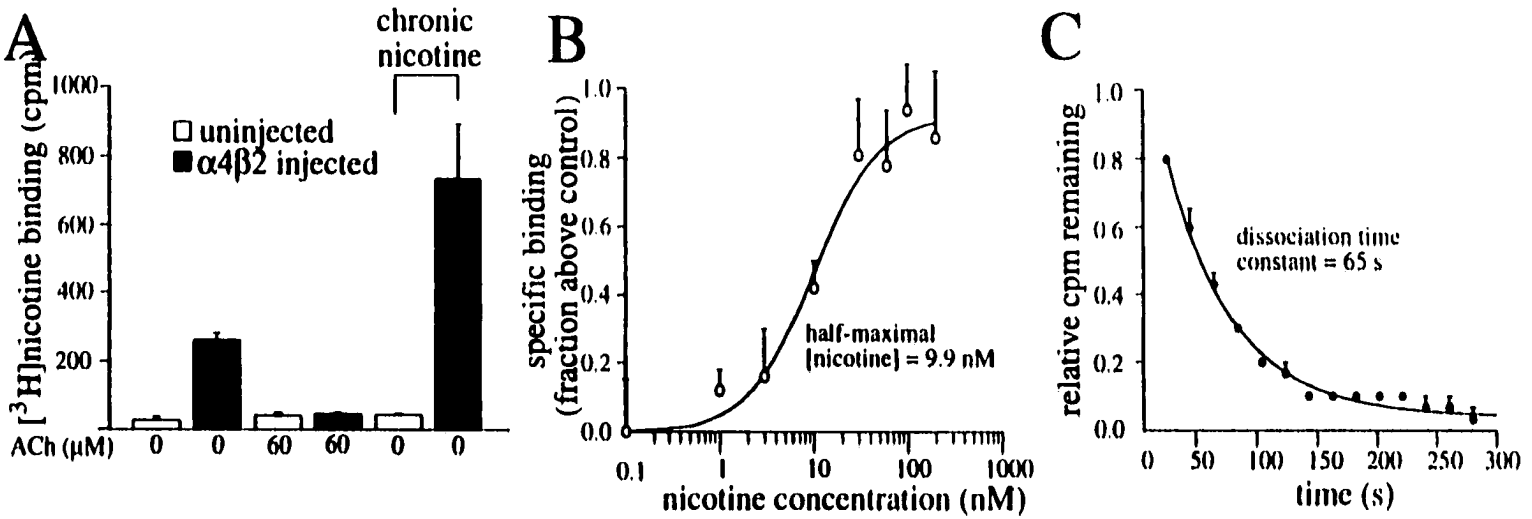


Figure 2. Upregulation of surface, α4β2 receptors after chronic nicotine incubation. *A*, Specific [^{3}H]nicotine binding to intact oocytes is upregulated by nicotine. Oocytes were uninjected (*open bars*) or injected (*filled bars*) with α4 and β2 subunit cRNAs. Twenty-four hours later, oocytes were placed in ND96+Ca with (*two right-most bars*) or without 60 nM nicotine. Surface binding assays were performed 24 hr later using 60 nM [^{3}H]nicotine. Nonradiolabeled ACh (60 μM) was used on some oocytes to determine nonspecific binding. Data are from five oocytes percondition. *B*, upregulation is nicotine concentration-dependent. α4β2-expressing oocytes were incubated in various nicotine concentrations for 24 hrs and then subjected to [^{3}H]nicotine binding assays using 60 nM [^{3}H]nicotine. A dose-response curve was then constructed from the relative increase in specific [^{3}H]nicotine binding at each nicotine incubation concentration. Data are plotted as the amount of specific [^{3}H]nicotine binding for oocytes incubated in nicotine compared with the specific [^{3}H]nicotine binding for control oocytes not incubated in nicotine (n = 4 - 9 oocytes per data point). *C*, nicotine dissociates rapidly from chronically incubated oocytes. α4β2-expressing oocytes were incubated in 300 nM [^{3}H]nicotine for 24 hrs. Dissociation of bound [^{3}H]nicotine was determined as described in Materials and Methods and plotted with respect to time. The *solid line* is an exponential fit to the data (n = 3 oocytes).

Protocols for Specific Experiments Involving Intact Oocytes

Specific binding of [^{3}H]nicotine to intact oocytes was used to measure three different parameters: (1) To measure the nicotine concentration necessary for half-maximal nAChR upregulation following chronic nicotine incubation, oocytes were chronically incubated (24 - 48 hr) in various concentrations of unlabeled nicotine. Assays were performed as described above using final [^{3}H]nicotine concentrations of 60 nM for $\alpha4\beta2$ nAChRs and 300 nM for $\alpha3\beta4$ nAChRs. (2) To determine the nicotine concentration necessary for half-maximal $\alpha4\beta2$ nAChR binding, assays were performed as described above; final [^{3}H]nicotine concentrations used ranged from 0.1 nM to 300 nM. For both the upregulation and equilibrium binding assays performed on intact oocytes, nonspecific binding was determined in the presence of 1000-fold excess of unlabeled acetylcholine. (3) To estimate the dissociation rate of bound [^{3}H]nicotine, assays were performed as described above using a final [^{3}H]nicotine concentration of 60 nM. At the end of the assay, the oocyte was suspended from the cut end of a pipette tip by light suction, washed sequentially in four different 2.5 ml wells containing ice-cold ND96+Ca, and then sequentially submerged (20 sec/well) into individual wells of a 96-well plate containing ND96+Ca and 60 μM unlabeled ACh (at room temperature). The contents of each well were then subjected to liquid scintillation counting.

Preparation of Total Homogenized Oocyte Membranes

For comparison, the concentrations of nicotine required for equilibrium binding and for receptor upregulation were also determined on oocyte membranes after homogenization. For equilibrium binding assays using cell homogenates, oocyte membranes were prepared as described (Corey et al., 1994). Briefly, oocyte membranes were isolated by centrifugation in 0.32 M sucrose in TE buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing protease inhibitors (200 mM phenylmethylsulfonyl-fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin) using 10-15 strokes of a tight-fitting

pestle in a chilled Dounce homogenizer. The homogenate was centrifuged twice at 4°C, 5 min, 1000 x *g* to remove cell debris, and the remaining supernatant fraction was homogenized at 4°C, 30 min, 100,000 x *g*. The membrane pellet was resuspended in homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA). Equilibrium [^{3}H]nicotine binding assays were performed by adding various concentrations of [^{3}H]nicotine (0.1 nM - 10 nM) to the resuspended membranes for 1 hr. Specific binding was determined in the presence of excess ACh (60 μM). The assay was terminated by filtration, using Whatman (Clifton, NJ) GFA filters soaked in polyethyleneimine (0.3% w/v). The filters were washed three times and subjected to liquid scintillation counting. Protein content was determined by the method of Bradford. For receptor upregulation, oocytes (16–20 per group) were incubated, before the assay, in various concentrations of nicotine (1-300 nM) for 24 hr. Membranes were prepared as described above, and specific binding was estimated using 60 nM [^{3}H]nicotine. Upregulation was expressed as the increase in binding relative to a control group.

RESULTS

α4β2 nAChRs Are Upregulated After Chronic Nicotine Treatment

To directly compare the dose-dependency for nicotine-induced upregulation of [^{3}H]nicotine binding sites with that of receptor desensitization, we performed binding assays (see Materials and Methods) on intact oocytes with and without chronic nicotine treatment. In a representative example (Fig. 2*A*), oocytes expressing α4β2 receptors showed 9.7-fold higher levels of surface [^{3}H]nicotine binding than uninjected oocytes. To determine the amount of specific surface [^{3}H]nicotine binding, we tested a subset of α4β2-expressing oocytes in the presence of excess unlabeled ACh. Unlabeled ACh reduced [^{3}H]nicotine counts to levels seen in uninjected control oocytes, permitting two conclusions: (1) that the majority of the [^{3}H]nicotine counts were the result of specific binding to nAChRs, and (2) because ACh is a membrane impermeant agonist (see

Whiteaker et al., 1998), that the majority of specific binding was associated with receptors on the plasma membrane (see Materials and Methods). Treatment of α4β2-expressing oocytes with 60 nM nicotine for 24 hr before the assay resulted in a 2.8-fold increase in [^{3}H]nicotine binding compared to α4β2-expressing oocytes not incubated in nicotine. This increase in binding caused by nicotine incubation was not seen in uninjected oocytes. Together, these data demonstrate that surface α4β2 nAChR expression is upregulated by chronic nicotine incubation. This result is likely due to an actual increase in the number of surface receptors since chronic agonist treatment fails to alter the apparent agonist binding affinity (Marks et al., 1983).

Similar [^{3}H]nicotine binding assays were repeated after 24 hr chronic nicotine treatment using nicotine concentrations ranging from 0.1 nM to 300 nM. Figure 2*B* shows a dose-response curve constructed from the relative increases in specific [^{3}H]nicotine binding after chronic nicotine treatment at eight different nicotine concentrations. Half-maximal upregulation of α4β2 nAChR expression was calculated to occur at nicotine incubation concentrations of 9.9 nM.

Chronic incubation using unlabeled nicotine raises a potential confound; that is, the unlabeled nicotine must fully dissociated from the receptor during the time of the binding assay. Otherwise, the number of [^{3}H]nicotine binding sites will be underestimated. To rule out this possibility and to determine the rate at which nicotine dissociates from α4β2 nAChRs, we performed an agonist dissociation assay (Fig. 2*C*). Oocytes were incubated in 300 nM [^{3}H]nicotine for 24 hr. In intact oocytes, dissociation (as described in Materials and Methods) of [^{3}H]nicotine from these receptors followed a single exponential time course with a time constant of 65 sec (Fig. 1*C*), which is similar to previous estimates (Marks and Collins, 1982; Lippiello et al., 1987). These data show that nicotine fully dissociates from α4β2 receptors within minutes and suggests that estimates of receptor upregulation following chronic nicotine treatment are likely not to be underestimated.

α4β2 nAChRs are Desensitized After Chronic Nicotine Treatment

To test the prediction that the concentrations of nicotine necessary for upregulation are similar to the nicotine concentrations necessary for desensitization, we measured desensitization functionally by two-electrode voltage clamp. Functional desensitization was assessed by measuring the fraction of activatable receptors remaining after a 24 hr nicotine incubation at concentrations ranging from 3 nM to 1000 nM. Changes in receptor responsiveness were estimated from whole-cell response amplitudes to nicotine test pulses applied near the EC_{50} for activation (10–20 μM; Fenster et al., 1997). Test pulses were administered before and after following incubation in nicotine (Katz and Thesleff, 1957; Feltz and Trautmann, 1982). Example test pulses are shown in Figure 3*A*. As illustrated in the *traces* from oocytes not treated with nicotine, control responses were often larger when measured 24 hr later (e.g., because of continual protein synthesis). To account for changes in whole-cell receptor responses, which were independent of nicotine and which occurred over the incubation time period, the fractional response remaining after 24 hr nicotine incubation was normalized to that of control oocytes not incubated in nicotine (see Materials and Methods).

Chronic incubation with increasing concentrations of nicotine resulted in a dose-dependent decrease in α4β2 receptor function (Fig. 3*C*). The data in Figure 3*B* also show the effect of removal of nicotine on the relative responses of these oocytes. There was a small increase in peak currents 1-2 hr after removing the oocytes from nicotine incubation, but almost no recovery thereafter. This increase in response may represent the return of some of the desensitized receptors to the activatable state in the first 2 hr after removal from nicotine incubation. Both the decreases in α4β2 receptor function after 24 hr nicotine incubation and the limited recovery following removal of nicotine are similar to results reported previously (Hsu et al., 1996).

Figure 3. Functional desensitization of α4β2 nAChRs following chronic nicotine incubation. *A*, Representative *traces* measured before and after incubation. Peak currents in α4β2-expressing oocytes induced by a nicotine test pulse (10 μM, 5 sec) were measured. The oocyte was then incubated for 24 hr in ND96+Ca in the absence (*top traces*) or presence (*bottom traces*) of 60 nM nicotine. Peak currents were remeasured immediately after removal from 24 hr incubation. To account for changes in basal receptor expression over the 24 hr incubation (*top traces*), the magnitude of nicotine-induced desensitization was defined as the ratio of the fractional response remaining after nicotine exposure to the fractional response remaining over the same period of time in the absence of nicotine (see Materials and Methods). *B*, Representative fractional nicotine-induced responses following 24 hr nicotine incubation. α4β2-expressing oocytes were incubated for 24 hr in control media or media containing 3, 30, 60, or 1000 nM nicotine. Measurement of peak currents before and after incubation and calculation of desensitization are described in *A*. For the 30 nM and 60 nM conditions, peak responses were also determined 2 and 24 hr after removal from nicotine. *C*, Inhibition dose-response curves constructed from fractional responses as described in *B*. Data are shown both uncorrected (*open circles*) and corrected (*filled circles*) for the amount of upregulation (i.e., relative increases in [^{3}H]nicotine binding) observed after 24 hr incubation in nicotine at the same concentration. The amount of upregulation at a given concentration is taken from the data in Figure 2*B*. The corrected values were calculated by dividing decreases in receptor function by the amount of upregulation (see text for details). The solid lines are logistic fits to mean data from which the half-maximal nicotine concentration for desensitization was (n = 3 to 32 oocytes per data point).

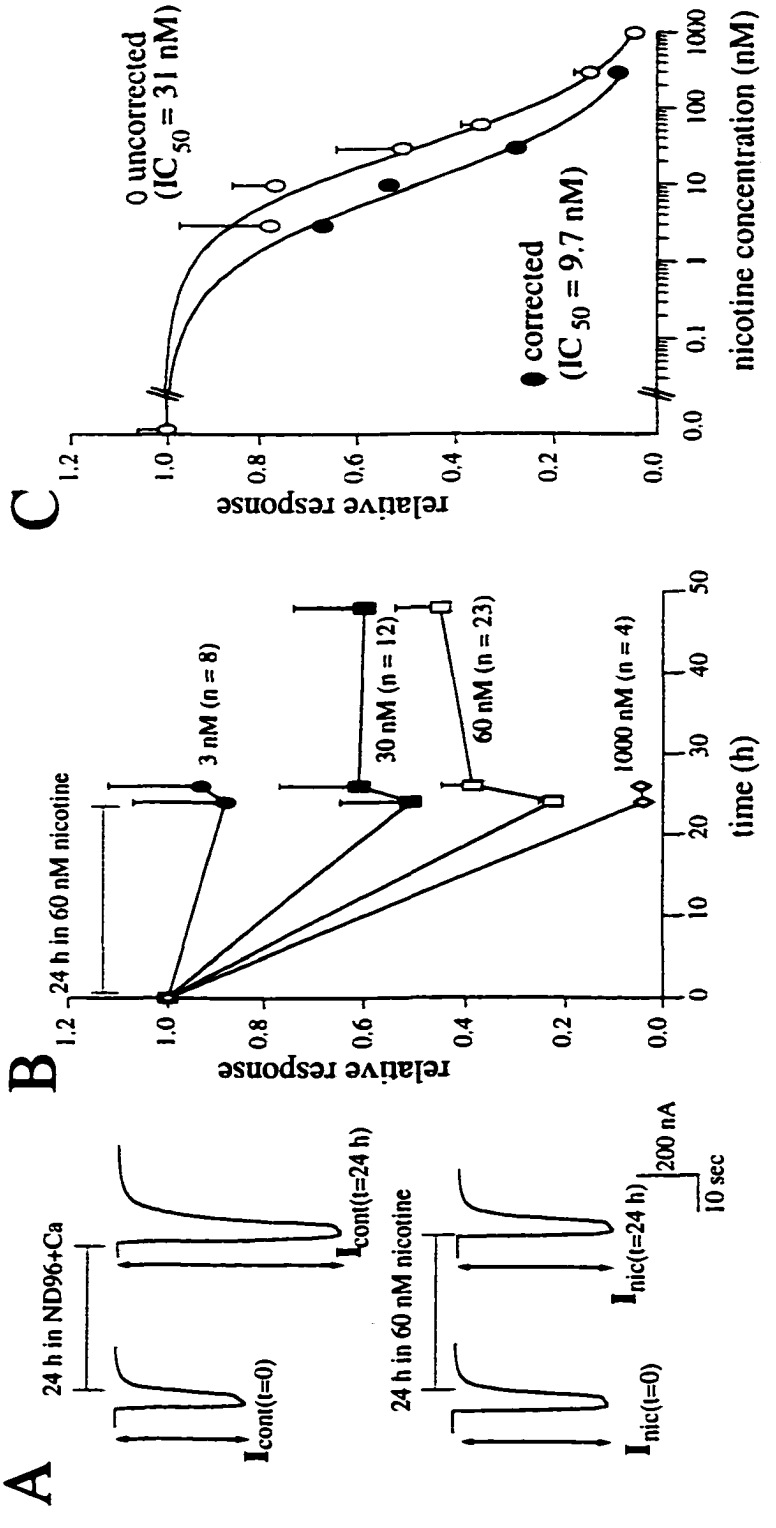


Figure 3*C* shows a dose-response curve constructed from the relative decrease in α4β2 receptor function after chronic nicotine treatment at six different nicotine concentrations (*open circles*). Half-maximal desensitization of α4β2 nAChRs was calculated to occur at nicotine incubation concentrations of 31 nM. Thus, it might be concluded that the concentrations of nicotine necessary to induce desensitization are approximately threefold higher than those required for upregulation. However, the calculated relative decrease in receptor function after nicotine exposure does not directly reveal the fraction of receptors that are activatable after incubation, because the total number of α4β2 receptors is upregulated during the 24 hr nicotine incubation. It is therefore necessary to correct for this change in receptor number. We normalized the relative response remaining after nicotine incubation by the amount of receptor upregulation observed at the particular nicotine incubation concentration (Fig. 2*B*). For example, after chronic incubation with 60 nM nicotine, the relative increase in [^{3}H]nicotine binding (*b*) was 178 ± 16%, and the fractional response remaining (*f*) was 0.36 ± 0.04 (i.e., 1.78 of the initial number of receptors were now responsible for 0.36 of the relative response). After correction (*f* / *b*), the fraction of activatable receptors remaining at the end of the 24 hr treatment with 60 nM nicotine was estimated to be 0.20. After correction of all concentration points, the half-maximal desensitization of α4β2 nAChRs was calculated to occur at nicotine incubation concentrations of 9.7 nM (Fig. 3*C*; *filled circles*). The similarity between the half-maximal values for upregulation of specific surface [^{3}H]nicotine binding to intact oocytes (see Fig. 2*B*) and functional desensitization is consistent with the idea that desensitization is a trigger for upregulation.

Upregulation and Desensitization of Lower Affinity nAChRs Are Correlated

If desensitization is a common trigger for upregulation of many different nAChRs, then a nAChR that has a lower affinity for nicotine-induced desensitization

should exhibit a comparably lower affinity for upregulation. To test this hypothesis, we examined the concentration-dependence of nicotine-induced desensitization and upregulation for α3β4 nAChRs, a receptor subtype with ~10-fold lower affinity for nicotine than α4β2 nAChRs (Fenster et al., 1997). As described for α4β2 nAChRs in Figure 3, functional desensitization was assessed from changes in receptor responsiveness following 24 hr incubation in nicotine at concentrations ranging from 30 nM to 10 μM. Representative *traces* for α3β4 expressing oocytes untreated or treated for 24 h in 3 μM nicotine are shown in Figure 4*A*. The relative responses remaining after incubation at two different concentrations, 1 μM and 10 μM, are shown in Figure 4*B*. These data were normalized to the responses of control oocytes not incubated in nicotine to factor in changes in receptor expression. Also, similar to the results shown for α4β2 nAChRs, little recovery of function from 24 hr nicotine incubation was evident in α3β4-expressing oocytes, even after 24 hr in the absence of nicotine. Similar experiments were repeated at several other nicotine incubation concentrations in order to obtain a dose-response curve for functional desensitization of α3β4 nAChRs caused by chronic nicotine treatment. The uncorrected half-maximal nicotine concentration for functional α3β4 desensitization was calculated to be 462 nM (Fig. 4C; *open circles*).

To determine the levels of nicotine necessary for α3β4 upregulation, we examined specific surface [^{3}H]nicotine binding to α3β4 nAChRs in intact oocytes following 24 hr nicotine incubation using concentrations ranging from 30 nM to 10 μM. An upregulation dose-response curve was constructed from the relative increases in [^{3}H] nicotine binding, and these data are plotted in Figure 4*D*. The half-maximal nicotine concentration for upregulation was estimated to be 215 nM. As described above for α4β2 nAChRs, we then used the relative increases in α3β4 binding at each nicotine concentration to correct for the relative response measured in the desensitization assays. The correction yielded a half-maximal nicotine concentration for functional α3β4 desensitization of 141 nM (Fig. 4*C*, *filled circles*). Thus, α3β4 receptors require

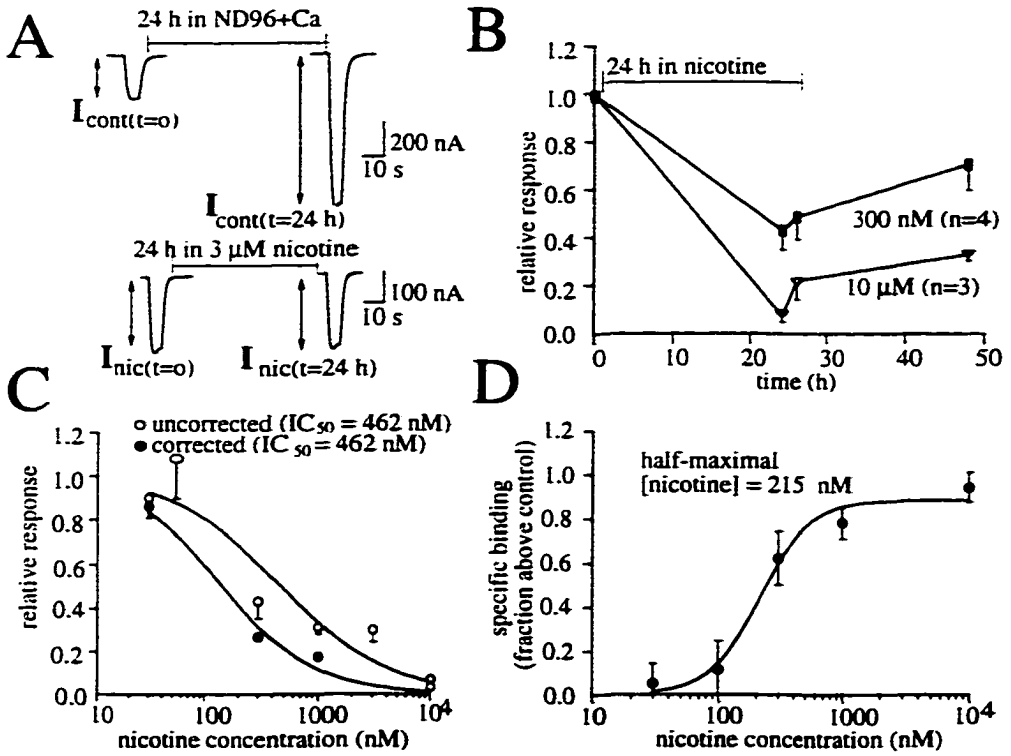


Figure 4. Desensitization and upregulation of α3β4 nAChRs. *A,* Representative traces measured before and after incubation. Experiments are the same as in Figure 3*A*, except that test pulses were performed with 60 μM nicotine and the 24 hr incubation was performed with 3 μM nicotine. *B,* Representative fractional nircotine-induced responses following 24 hr nicotine incubation. α3β4-expressing oocytes were incubated for 24 hr in control media or media containing 300 nM or 10 μM nicotine. Measurement of peak currents before and after incubation, and calculation of desensitization are described in Figure 3*A*. Peak responses were also determined 2 and 24 hr after removal from nicotine. *C,* Inhibition dose-response curves constructed from fractional responses as described in *B*. Data are shown both uncorrected (*open circles*) and corrected (*filled circles*) for the amount of upregulation (i.e., relative increases in [^{3}H]nicotine binding) observed following 24 hr incubation in nicotine at the same concentration (see Fig. 3C). The amount of upregulation at a given concentration is taken from the data in Figure 4*D*. The solid lines are logistic fits to mean data from which the half-maximal nicotine concentration for desensitization was (*n* = 3 to 15 oocytes per data point). *D,* Upregulation is nicotine concentration-dependent. [^{3}H] Nicotine binding assays were performed as described in Figure 2*B*. Data are plotted as the amount of specific [^{3}H]nicotine binding for oocytes incubated in nicotine compared with the specific [^{3}H]nicotine binding for control oocytes not incubated in nicotine (*n* = 4 - 9 oocytes per data point).

~10-fold higher nicotine concentrations to induce half-maximal desensitization than α4β2 receptors, and this shift is paralleled by a comparable shift in the nicotine concentrations necessary for upregulation.

Receptors That Do Not Recover from Desensitization Upregulate After Brief Applications of Nicotine

Previously, we produced a mutant α4 subunit in which a putative PKC phosphorylation site was eliminated ($S^{336}A$). Briefly, when this subunit is coexpressed with a wild-type β2 subunit, it forms receptors in oocytes that exhibit many functional properties similar to the wild-type α4β2 receptors. For example, (1) dose-response relationships for nicotine-induced activation estimate an EC_{50} value of 13 μM, which is similar to the estimated EC_{50} value for wild-type receptors (15 μM; Fenster et al., 1997); and (2) rates into the desensitized state (induced by 2 min applications of 10 μM nicotine) are the same as for wild-type α2β4 receptors. However, $\alpha4^{S336}A\beta2$ receptors are different from wild-type receptors in one way that is important for the present studies; whereas wild-type α4β2 receptors recovered fully from desensitization (with a time constant of ~43 min), $\alpha4^{S336}A\beta2$ receptors showed <20% total recovery (Fenster et al., 1999). We reasoned that if desensitization is the trigger for upregulation, then we should be able to make specific predictions regarding upregulation in the mutant that would be different than for wild-type α4β2 receptors.

Because the mutant α4β2 receptor does not readily return to the activatable state after desensitization, one prediction is that once mutant receptors are desensitized by nicotine, then nicotine will no longer be required to produce upregulation. This would not be true of wild-type receptors, which would recover from desensitization in the absence of nicotine. To test this prediction, we subjected oocytes injected with either wild-type or mutant receptors to periodic nicotine treatment. Specifically, the oocytes were incubated for 1 hr every 12 hr in 60 nM nicotine. The rationale was that 1-hr treatment

would desensitize both receptor types, but the wild-type receptors would recover in the absence of nicotine during the subsequent hour; nicotine was added for 1 hr at 12 hr intervals in order to desensitize any new receptors that had been inserted during the assay. Data obtained from oocytes in the periodically incubated condition were compared with oocytes that were untreated or incubated continuously for 48 hr in 60 nM nicotine. Wild-type nAChRs were not significantly upregulated after periodic treatment. In contrast, the mutant nAChRs were upregulated to levels comparable with that seen with continuous nicotine treatment (Fig. 5*C*).

A potential explanation for these data is that nicotine remains bound to the mutant receptors and thus the "periodic" treatment is in fact "continuous" treatment. To rule out this possibility, we examined the dissociation rate of [^{3}H] nicotine from $\alpha4^{S336A}\beta2$ receptors. The dissociation time constant was 69 sec (data not shown), which is similar to that observed for wild-type $\alpha4\beta2$ nAChRs (Fig. 2*C*). The most straightforward interpretation of the periodic treatment data is that, at least in the case of the mutant receptor, the unoccupied desensitized state is sufficient to induce upregulation.

Mutant α4β2 nAChRs Have a Higher Apparent Affinity for Nicotine

In addition to the finding that periodic treatment with nicotine could upregulate mutant receptors, continuous incubation with 60 nM nicotine for 48 hr resulted in a greater amount of mutant nAChR upregulation (~150%) compared to wild-type receptor nAChR upregulation (~80%; Fig. 5*A*). These data imply that the mutant nAChRs may have a higher affinity for nicotine (i.e., upregulation would occur at lower agonist concentrations). To test this idea, we replotted the [^{3}H]nicotine saturation data presented in Figure 1*A* for wild-type $\alpha4\beta2$ nAChRs measured in intact oocytes (Fig. 5*B*; *filled circles*). The concentration of nicotine required for half-maximal binding to wild-type receptors in intact oocytes was estimated to be 11 nM. In comparison, the

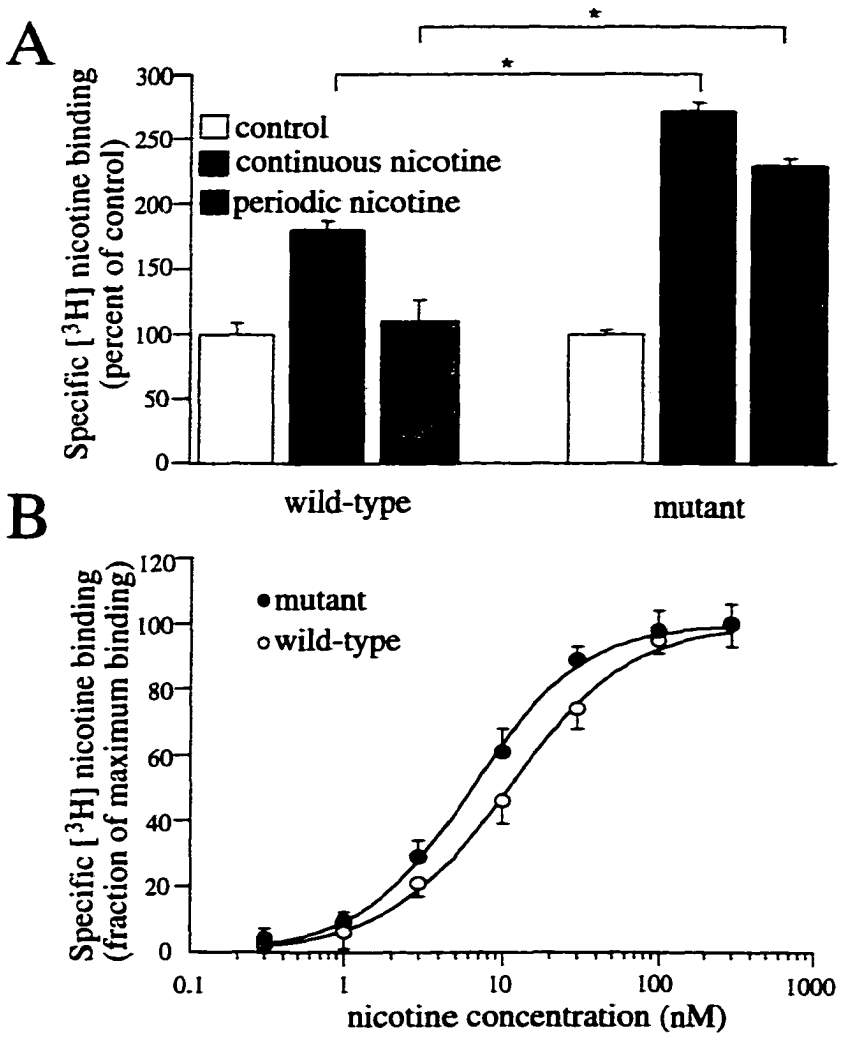


Figure 5. Upregulation and equilibrium binding to mutant ($S^{336}A$) α4β2 receptors. *A,* Mutant, but not wild-type, α4β2 receptors are upregulated by periodic nicotine treatment. Expressing oocytes were incubated continuously for 48 hr in ND96+Ca (*open bars*), continuously for 48 hr in 60 nM nicotine (*filled bars*), or periodically (*hatched bars*; 1 hr in 60 nM nicotine every 12 hr for 48 hr). Specific, surface [H]nicotine binding to intact oocytes was then measured for 1 hr using 60 nM [3H]nicotine. Data are from five to seven oocytes per condition (* $p < 0.05$; unpaired t test). *B,* Equilibrium binding to intact oocytes expressing wild-type (*filled circles*) or mutant ($S^{336}A$; *open circles*) α4β2 receptors. Assays were performed for 1 hr using [^{3}H]nicotine concentrations from 0.1 to 300 nM. Dose-response curves were constructed from the amount of specific [^{3}H]nicotine binding normalized to maximal [^{3} H]nicotine binding (n = 5 - 6 oocytes per data point).

concentration of nicotine required for half-maximal binding to mutant $\alpha4^{S336}A\beta2$ receptors in intact oocytes was estimated to be 6 nM. Thus, mutant $\alpha4\beta2$ receptors are more readily upregulated than wild-type nAChRs, and this difference is correlated with a shift in equilibrium nicotine binding.

Equilibrium Binding to α4β2 nAChRs is Altered by Membrane Homogenization

The present data suggest that upregulation and desensitization are closely related phenomena. Previous estimates of these parameters have not revealed such a close association between the concentration of nicotine necessary for upregulation and that which produces desensitization (Peng et al., 1994; Whiteaker, 1998). In most of these studies, however, estimates of the potency of nicotine for desensitization was obtained from equilibrium [^{3}H]nicotine binding. Therefore, one possibility for the apparent concentration mismatch between upregulation and desensitization is that equilibrium binding studies measure receptors in an altered state(s) from that encountered when measuring functional desensitization. In the present study, the half-maximal value obtained for equilibrium binding of [^{3}H]nicotine to $\alpha4\beta2$ nAChRs in intact oocytes (11 nM; Fig. 5*B*) is comparable with that for desensitization (10 nM; Fig. 3*C*). Together with our observation that the number of surface agonist binding sites and functional channels are correlated (Fig. 1*B*), these data suggest that equilibrium [^{3}H]nicotine binding to intact surface receptors and functional desensitization are likely measuring the same population of $\alpha4\beta2$ nAChRs in the same state(s).

Another explanation for large differences between previously observed dose-response curves for receptor upregulation and equilibrium binding is that the majority of binding studies have been performed on membrane homogenates. Such preparations may differ from surface binding in intact preparations because of contributions from non-surface receptors and/or changes in the biochemical state of the receptor (Wonnacott, 1987). To test this hypothesis, we performed saturation [^{3}H]nicotine binding assays on

cell lysates obtained from oocytes expressing α4β2 nAChRs and compared the results to those obtained from the intact oocyte experiments (Fig. 6). Following membrane homogenization, the dose-response curve for equilibrium nicotine binding was shifted significantly to the left; the concentration of nicotine for half-maximal binding was estimated to be 0.4 nM. These data demonstrate that the apparent affinity of nAChRs for nicotine is altered after membrane homogenization (see also Whiteaker et al., 1998), implying that equilibrium binding may not always accurately reflect *in vivo* receptor desensitization. In addition, at near saturating concentrations of [^{3}H]nicotine (60 nM), specific binding to membrane homogenates was ~sixfold greater than for surface binding to intact oocytes, implying that >80% of the binding in membrane homogenates was to intracellularly localized receptors. Together, the increase in both the apparent affinity and the number of receptors after membrane homogenization argues that most nAChRs in oocytes exist in a high-affinity intracellular pool, with a smaller receptor population on the cell surface that has a lower apparent affinity for nicotine.

Upregulation of α4β2 nAChRs in Total Membrane Homogenates

If desensitization of surface α4β2 receptors is the trigger for upregulation of intracellular as well as surface receptors (Whiteaker et al., 1998), then the half-maximally effective concentration for upregulation of both populations should be the same. If, however, these populations are regulated independently, then their concentration requirements for upregulation may be different. As we have shown for surface receptors, upregulation occurs at concentrations that induce desensitization. Because intracellular receptors (the majority of binding sites on homogenized membranes) appear to have a higher apparent affinity than surface receptors, it might be predicted that upregulation will occur at lower concentrations of nicotine. However, after chronic (24 hr) exposure to nicotine, we observed that upregulation of specific [^{3}H]nicotine binding to isolated membranes required higher agonist concentrations than those necessary for upregulation

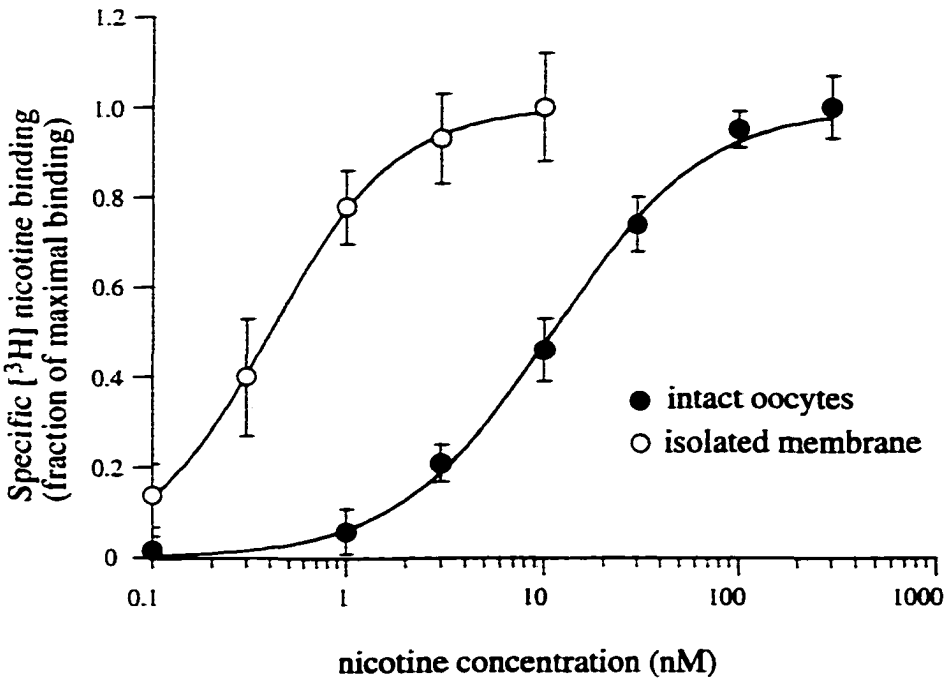


Figure 6. Equilibrium binding to intact and homogenized oocyte membranes. α4β2-expressing oocytes from the same oocyte batch were subjected to membrane-intact (*filled circles*) or membrane-homogenate (*open circles*) [^{3}H]nicotine binding assays. Assays were performed for 1 hr using [^{3}H]nicotine concentrations from 0.1 to 300 nM. Dose-response curves were constructed from the amount of specific [^{3}H]nicotine binding normalized to maximal [^{3}H]nicotine binding (n = 5 - 6 oocytes per data point).

of surface receptors. The half-maximal concentration of nicotine for upregulation of intracellular nAChRs was 58 nM (Fig. 7). Thus, as others have observed previously, there is a discrepancy between the concentrations required for half-maximal equilibrium binding and those necessary for upregulation in homogenized membranes (Peng et al., 1994; Bencherif et al., 1995; Warpman et al., 1998; Whiteaker et al., 1998).

DISCUSSION

In the present study, we provide several lines of evidence to suggest that, for $\alpha4\beta2$ nAChRs on the surface of oocytes, receptor upregulation is directly related to receptor desensitization: (1) the half-maximal value for nicotine-induced upregulation is equal to the half-maximal effective concentrations of nicotine required for both functional receptor desensitization and equilibrium binding to surface nAChRs in intact oocytes; (2) the half-maximal value for upregulation of $\alpha3\beta4$ nAChRs is ~10-fold higher than for $\alpha4\beta2$ nAChRs, and this shift is mirrored by a 10-fold shift in the half-maximal concentration necessary for functional desensitization; and (3) mutant $\alpha4\beta2$ receptors, for which recovery from the desensitized state does not readily occur (Fenster et al., 1999), can be upregulated in the absence of nicotine. Additionally, we find that much lower concentrations of [^{3}H]nicotine are needed for half-maximal equilibrium binding to receptors after membrane homogenization. These latter data may account for some of the discrepancies between apparent affinities for equilibrium binding and upregulation observed here and in other systems (Peng et al., 1994; Whiteaker et al., 1998).

$\alpha4\beta2$ nAChR Equilibrium Binding and Functional Desensitization

Based on the Katz and Thesleff (1957) cyclical model of desensitization, as illustrated below, nAChRs may exist either in activatable states *R* or higher affinity desensitized states *D*, where *L* is equal to the ratio of desensitized to activatable receptors D/R.

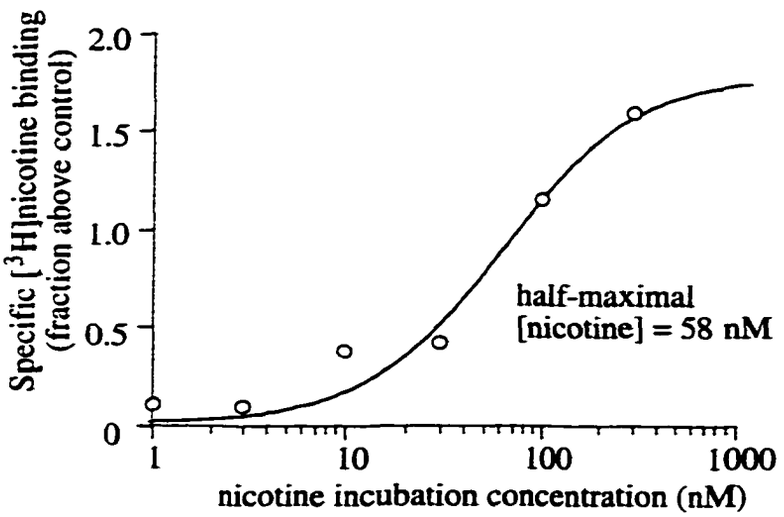
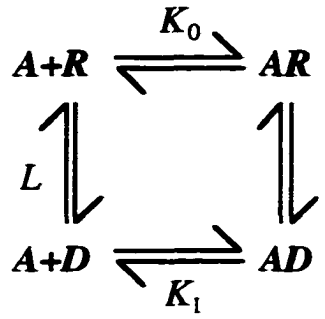


Figure 7. Upregulation of total, α4β2 receptors after chronic nicotine incubation. Specific [^{3}H]nicotine binding to homogenized membranes is upregulated by nicotine. Oocytes were injected with α4 and β2 subunit cRNAs. Twenty-four hours later, oocytes were placed in ND96+Ca in the absence or presence of various concentrations of nicotine for 24 hr. After preparation of homogenized membranes, binding assays were performed using 60 nM [^{3}H]nicotine. The plot shows a dose-response curve constructed from the relative increase in specific [^{3}H]nicotine binding to homogenized membranes at each nicotine incubation concentration. Data are plotted as the amount of specific [^{3}H]nicotine binding for oocytes incubated in nicotine compared with the specific [^{3}H]nicotine binding for control oocytes not incubated in nicotine (n = 16 - 20 oocytes per data point).



Because the affinity of nicotine is higher for the desensitized state compared with the activatable state (i.e., $K_0 >> K_I$; Katz and Thesleff, 1957; Feltz and Trautmann, 1982), prolonged nicotine exposure should stabilize receptors in the agonist-bound desensitized state *AD*. Based on this model, the apparent affinity of the desensitized state can be estimated using either measures of functional desensitization or equilibrium binding (Lippielo et al., 1987; Grady et al., 1994). Consistent with this idea, we and others (Higgins and Berg, 1988; Grady et al., 1994) find that apparent affinities for equilibrium binding and functional desensitization are similar.

After chronic exposure to low concentrations of nicotine, our functional assessment of α4β2 nAChR desensitization measures the relative fraction of activatable receptors R / R_{max} remaining (Feltz and Trautmann, 1982):

$$\frac{R}{R_{max}} = \frac{1+L}{1+L(1+[A]/K_I)} \qquad (2)$$

At equilibrium, [^{3}H]nicotine binding will measure the fraction f of total receptors in the desensitized state *AD*:

$$fAD = \frac{1}{1+\frac{K_I}{[A]}(1+\frac{1}{L})} \qquad (3)$$

Both assessments of the potency of nicotine for inducing desensitization will be influenced by the ratio of desensitized to activatable receptors L (Marks et al., 1996); thus, the half-maximal effective concentrations of nicotine that we have calculated are related, but not equal, to the affinity of nicotine for the desensitized state. The true affinity of the desensitized state K_I can be determined only if an independent estimate of L is obtained (Lippiello et al., 1987). More importantly for the present study, Equations 2 and 3 predict the same half-maximal agonist values for functional desensitization and equilibrium binding if K_I and L remain constant for both types of measurements. The most straightforward empirical method for doing this is to determine these two half-maximal concentrations under comparable conditions. Measuring both specific radiolabeled binding to surface nAChRs in intact oocytes and functional desensitization in the same oocytes (or oocytes from the same batch) is one such approach.

It has been hypothesized that chronic nicotine-induced upregulation in the number of high affinity ($\alpha4\beta2$) binding sites in the CNS is a consequence of receptor desensitization (Marks et al., 1983; Schwartz and Kellar, 1985). From the model, upregulation will be directly related to occupation of the desensitized state by nicotine, and therefore the concentration of nicotine required for upregulation can be predicited from Equation 3. Then, if the desensitization hypothesis is correct, the half-maximal nicotine concentrations for both upregulation and equilibrium [^{3}H]nicotine binding should be the same. Our data for nAChRs expressed on the surface of intact oocytes are consistent with this hypothesis.

Receptor Pools and Apparent [^{3}H]Nicotine Equilibrium Binding Affinities

We observed a > 20-fold decrease in the half-maximal [^{3}H]nicotine concentration necessary for $\alpha4\beta2$ nAChR equilibrium binding after membrane homogenization compared with that measured for surface receptors in intact oocytes. In addition, membrane homogenization revealed a population of receptors ($\approx$ 80% of total receptors)

not measured during surface binding assays on intact oocytes. From these observations, we may conclude that the majority of nAChRs are intracellular (Whiteaker et al., 1998), with a higher apparent affinity for nicotine than surface receptors. Why then is the surface pool of receptors not detected as a lower affinity component (~20%) of [^{3}H] nicotine binding to homogenized membranes? Aside from the potential difficulty in distinguishing this component, it may be that membrane homogenization affects the integrity of surface receptors, allowing them to enter higher affinity states.

From cyclical models of desensitization, there are two ways of altering the apparent agonist binding affinity (Marks et al., 1996): a change in the microscopic affinity constant K_1 or a change in the ratio of desensitized to activatable receptors L. Because the dissociation rate of nicotine from intact oocytes is similar to that obtained after membrane homogenization (Marks and Collins, 1982), the microscopic affinity of the desensitized state K_1 is likely unaffected by cell lysis. Thus, the effect of membrane homogenization may be in part explained by a shift in L, the initial fraction of receptors in the desensitized state. If this shift favors more receptors in the desensitized state, then the apparent binding affinity will increase and approach the microscopic affinity of the desensitized state (Marks et al., 1996).

We suggest that some of the differences in the apparent affinity of nicotine for receptors on internal and/or homogenized membranes compared with intact surface receptors are the result of altered biochemical regulation. Consistent with this idea, it has been shown that recovery from desensitization of $\alpha4\beta2$ nAChRs is enhanced by PKC activation and phosphatase inhibition (Eilers et al., 1997; Fenster et al., 1999). Because recovery from desensitization likely proceeds via the transition from desensitized to activatable receptors, then the allosteric constant L will be increased by phosphorylation, which will in turn lead to a decrease in the apparent binding affinity (Equation 3). In support of this suggestion, we report here that mutant $\alpha4\beta2$ receptors have an approximate twofold higher apparent [^{3}H] nicotine binding affinity than wild-

type receptors. Because the rate of nicotine dissociation from the desensitized state is not changed in mutant receptors, the increased affinity of mutant channels is predicted from the decrease in L that likely results from the slowed rate of recovery from desensitization in the mutant channel (Fenster et al., 1999).

Based on the above interpretation, we suggest that, in contrast to wild-type $\alpha4\beta2$ nAChRs, mutant receptors become trapped in the unbound desensitized state, after chronic exposure to nicotine. It follows that because the mutant receptor can upregulate in the absence of ligand (Fig. 5), the unoccupied desensitized state may be sufficient for inducing upregulation under certain circumstances. This result is consistent with the finding that chronic PKC inhibition, which alone can downregulate $\alpha4\beta2$ receptor function (Eilers et al., 1997), presumably by shifting more receptors into the desensitized state (Fenster et al., 1999), can cause upregulation (Gopalakrishnan et al., 1997) in the absence of nicotine.

Is Desensitization a General Trigger for Upregulation of nAChRs?

If upregulation of both surface and intracellularly localized receptors is triggered through a common mechanism, e.g., the interaction of nicotine with cell surface receptors (Whiteaker et al., 1998), then upregulation of both pools should have the same dependency on nicotine concentration. However, the half-maximal concentration of nicotine required for upregulation of the intracellular pool (in homogenized membranes) was higher (~60 nM) than that necessary for upregulation of surface nAChRs (~10 nM). These data imply that, unlike surface nAChRs, upregulation of intracellular $\alpha4\beta2$ receptors in oocytes is not initiated through an interaction with the desensitized state of surface nAChRs. Moreover, if the apparent binding affinity for nicotine (~400 pM) in homogenized membranes largely reflects desensitized intracellular receptors, it is very unlikely that upregulation is mediated via occupation of the desensitized state of this pool of receptors. Based on our suggestion for surface receptors, it may be that

intracellular nAChRs are shifted to a higher affinity state by membrane homogenization. However, there is only a twofold difference in the apparent agonist binding affinities to intracellular chick $\alpha4\beta2$ nAChRs between intact M10 cells and isolated membranes (Whiteaker et al., 1998), this seems improbable. Thus, in the case of intracellular receptors, we, like many others, are left to explain why greater than saturating concentrations of nicotine are required for receptor upregulation (Peng et al., 1994; Bencherif et al., 1995; Warpman et al., 1998; Whiteaker et al., 1998). One explanation is that nicotine directly (i.e., in a nonreceptor-mediated manner) interferes with processes that regulate the number of nAChRs. To answer this question, it will be necessary to more fully understand the factors that control the movement of receptors between functional and silent surface pools and intracellular pools.

Conclusions

The overall problem of how chronic nicotine alters the number and function of nAChRs remains unresolved, however, we suggest that desensitization plays an important role in upregulation of the population of receptors on the plasma membrane. Although other factors must be taken into account *in vivo* (Rowell and Li, 1997), the relationship between desensitization and upregulation should be useful for predicting the long-term consequences of tobacco-related levels of nicotine on different subtypes of nAChRs in the CNS.

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REGULATION OF α4β2 NICOTINIC RECEPTOR DESENSITIZATION BY CALCIUM AND PROTEIN KINASE C

by

CATHERINE P. FENSTER, MATTHEW L. BECKMAN, JULIE C. PARKER, ELISE B. SHEFFIELD, TERRI L. WHITWORTH, MICHAEL W. QUICK AND ROBIN A.J. LESTER

Molecular Pharmacology (1999) 55:432-443

ABSTRACT

Neuronal nicotinic acetylcholine receptor (nAChR) desensitization is hypothesized to be a trigger for long-term changes in receptor number and function observed after chronic administration of nicotine at levels similar to those found in persons who use tobacco. Factors that regulate desensitization could potentially influence the outcome of long-lasting exposure to nicotine. The roles of Ca^{2+} and protein kinase C (PKC) on desensitization of α4β2 nAChRs expressed in *Xenopus laevis* oocytes were investigated. Nicotine-induced (300 nM; 30 min) desensitization of α4β2 receptors in the presence of Ca^{2+} developed in a biphasic manner with fast and slow exponential time constants of τ_f = 1.4 min (65% relative amplitude) and τ_s = 17 min, respectively. Recovery from desensitization was reasonably well described by a single exponential with τ_{rec} = 43 min. Recovery was largely eliminated after replacement of external Ca^{2+} with Ba^{2+} and slowed by calphostin C (τ_{rec} = 48 min), an inhibitor of PKC. Conversely, the rate of recovery was enhanced by phorbol-12-myristate13-acetate (τ_{rec} = 14 min), a PKC activator, or by cyclosporin A (with τ_{rec} = 8 min), a phosphatase inhibitor. α4β2 receptors containing a mutant α4 subunit that lacks a consensus PKC phosphorylation site exhibited little recovery from desensitization. Based on a two-desensitized state cyclical model, it is proposed that after prolonged nicotine treatment, α4β2 nAChRs accumulate in a "deep" desensitized-state, from which recovery is very slow. We suggest that PKC-dependent phosphorylation of α4 subunits changes the rates governing the transitions from "deep" to "shallow" desensitized conformations and effectively increases the overall rate of recovery from desensitization. Long-lasting dephosphorylation may underlie the "permanent" inactivation of α4β2 receptors observed after chronic nicotine treatment.

INTRODUCTION

The family of neurotransmitter-gated ion channels is responsible for fast synaptic transmission in the peripheral and central nervous system (CNS) (Barnard et al., 1987; Unwin, 1989; Betz, 1990). Functional regulation of these receptors by second messenger systems has often been examined with respect to their involvement in neuronal plasticity (Swope et al., 1992; Smart, 1997). How receptor modulation contributes to possible dysfunction of receptors in disease states is less well understood. It is possible that various intracellular mechanisms required to regulate receptors under normal circumstances confer long-lasting changes during abnormal conditions. Such receptor regulation could underlie the "functional inactivation" of neuronal nicotinic acetylcholine receptors (nAChRs) that occurs during and after chronic exposure to nicotine (Lukas, 1991; Peng et al., 1994).

Neuronal nAChRs are amenable to a variety of physiologically relevant forms of regulation. The number and/or function of receptors can be increased through both cAMP-dependent and independent mechanisms (Margiotta et al., 1987; Gurantz et al., 1993), by protein kinase C (PKC) (Downing and Role, 1987), by the neurotransmitters vasoactive intestinal peptide (Gurantz et al., 1994; Cuevas and Adams, 1996) and Substance P (Role, 1984), by changes in the extracellular concentration of Ca^{2+} (Mulle et al., 1992b; Vernino et al., 1992; Galzi et al., 1996) and through interactions with the cytoskeleton (Bencherif and Lukas, 1993). In addition, the permeation of Ca^{2+} through nAChR channels (Vernino et al., 1992) could activate intracellular cascades or other ion channels (Mulle et al., 1992a) and potentially induce changes in the phosphorylation states of specific nAChR subunits (Vijayaraghavan et al., 1990; Nakayama et al., 1993; Moss et al., 1996). The long-term functional consequences of such post-translational modifications remain largely unexplored.

Because of nAChR subunit diversity (McGehee and Role, 1995; Colquhoun and Patrick, 1997), their biochemical regulation may occur in a subtype-specific manner.

Although it is unclear which subtypes of nAChRs predominate in CNS function, receptors containing α4 and β2 subunits contribute to a majority of the high affinity nicotine binding sites in brain (Whiting and Lindstrom, 1988; Flores et al., 1992). Furthermore, nicotine at related to tobacco use (Benowitz et al., 1989) both activates and desensitizes α4β2 nAChRs (Hsu et al., 1995; Fenster et al., 1997); desensitization may initiate the upregulation of nAChR number that occurs during chronic nicotine exposure (Wonnacott, 1990; Schwartz and Kellar, 1995). Therefore, factors that regulate α4β2 nAChR function, especially those that modulate desensitization, may contribute to the long-term effects of nicotine on nAChR number and function. In this study, we investigate second messenger modulation of α4β2 nAChRs expressed in *Xenopus laevis* oocytes. We show that the predominant role of Ca^{2+} and PKC is to regulate the rate of recovery from desensitization.

MATERIALS AND METHODS

Expression of Functional nAChRs in Xenopus *Oocytes*

Detailed procedures for preparation of oocytes have been described elsewhere (Quick and Lester, 1994). Briefly, oocytes were defolliculated and maintained at 18°C in incubation medium containing ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4), 50 mg/ml gentamicin, and 5% horse serum. Subunit cRNAs were synthesized *in vitro* (machine message; Ambion, Austin, TX) from linearized plasmid templates of rat cDNA clones. A mutant α4 subunit ($\alpha4S^{336}A$) was created in which a consensus PKC phosphorylation site (serine 336) was mutated to alanine (pALTER 1; Promega, Madison, WI). The mutation was verified by sequencing. Oocytes were injected with between 5 and 25 ng/subunit/oocyte; α and β subunits were injected in 1:1 ratios.

Electrophysiological Recording

Whole-cell currents were measured at room temperature (20–25°C), 24-96 hr after RNA injection, with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) in a standard two-microelectrode, voltage-clamp configuration. Electrodes were filled with 3 M KCl and had resistances of 0.5 to 2 MΩ. Oocytes were clamped between -40 and -65 mV and superfused continuously in media containing 1.8 mM Ca^{2+} (control condition). In some experiments 1.8 mM Ba^{2+} was substituted for Ca^{2+}. In some of the early experiments, membrane-permeable drugs were applied by extracellular incubation. In the majority of experiments, drugs (25 nl) were injected into the oocytes 10 to 30 min prior to experimentation, so as to avoid direct extracellular effects of protein kinase activators/inhibitors on nAChR channels (Reuhl et al., 1992). The approximate final concentrations were: phorbol-12-myristate-13-acetate (PMA), 200 nM to 2 μM; cyclosporin A, 500 nM to 1 μM; and calphostin C, 200 nM. Control incubations or injections were performed with vehicle solutions: 0.125% dimethyl sulfoxide (DMSO) for calphostin C and PMA treatments. Agonist-containing solutions were gravity-fed via a six-way manual valve (Rainin Instruments, Woburn, MA) to the oocyte in the recording chamber. Solution exchange considerations are discussed in Fenster et al. (1997). All salts and drugs were obtained from Sigma (St. Louis, MO). All currents were recorded on a chart recorder and on an 80486-based computer with AxoScope software (Axon Instruments) after 50-100 Hz low-pass filtering at a digitization frequency of 200 Hz. For slowly desensitizing responses, peak currents were assessed on-line from the digital readout amplifier

Criteria for Data Selection

For accurate voltage-clamp, and to limit the activation of the endogenous Ca^{2+}-activated Cl^- current in oocytes, nicotine-induced currents greater than 3 μA were not included in the data analysis; initial current amplitudes less than 50 nA were also

excluded. Additionally, responses were at least twofold greater than the holding current, and the holding current at a given membrane potential was less than 100 nA. These criteria applied to all nAChR currents activated at the EC_{50} concentration. Various desensitization parameters were estimated as described previously (Fenster et al., 1997). For the majority of experiments, desensitization was studied using the methods of Katz and Thesleff (1957) and Feltz and Trautmann (1982). Briefly, the fraction of activatable receptors before, during, and after a 30 min exposure to 300 nM nicotine was assessed from the amplitude of repetitively applied test pulses (≈ 5 sec; 10 - 20 μM nicotine; interpulse interval = 5 min). The respective time courses of desensitization onset and recovery were estimated from exponential fits to the test pulse amplitude during and after the 300 nM nicotine application. Fits were sometimes constrained so that steady-state desensitization could not be less than zero. For means of comparison across different conditions, the magnitude of desensitization was calculated as the ratio $(I_{con} - I_{final}) / I_{con}$, where I_{con} is the control test pulse amplitude and I_{final} is its amplitude at the end of the 30 min nicotine application. In a few experiments, desensitization onset and magnitude were determined from the response to a 2-3 min application of 10 μM nicotine. For statistical comparison of mean data, weighted means *t* tests (for unpaired comparisons) and paired *t* tests (for paired comparisons) were performed. Comparisons of exponential fits were by nonlinear regression analysis using SPSS software (SPSS for Windows; Rel. 8.0.0. 1997; SPSS Inc., Chicago, IL). All data are expressed as the mean ± SEM. Kinetic models of receptor desensitization were constructed using ScOP (Simulation Resources Inc., Berrien Springs, MI).

RESULTS

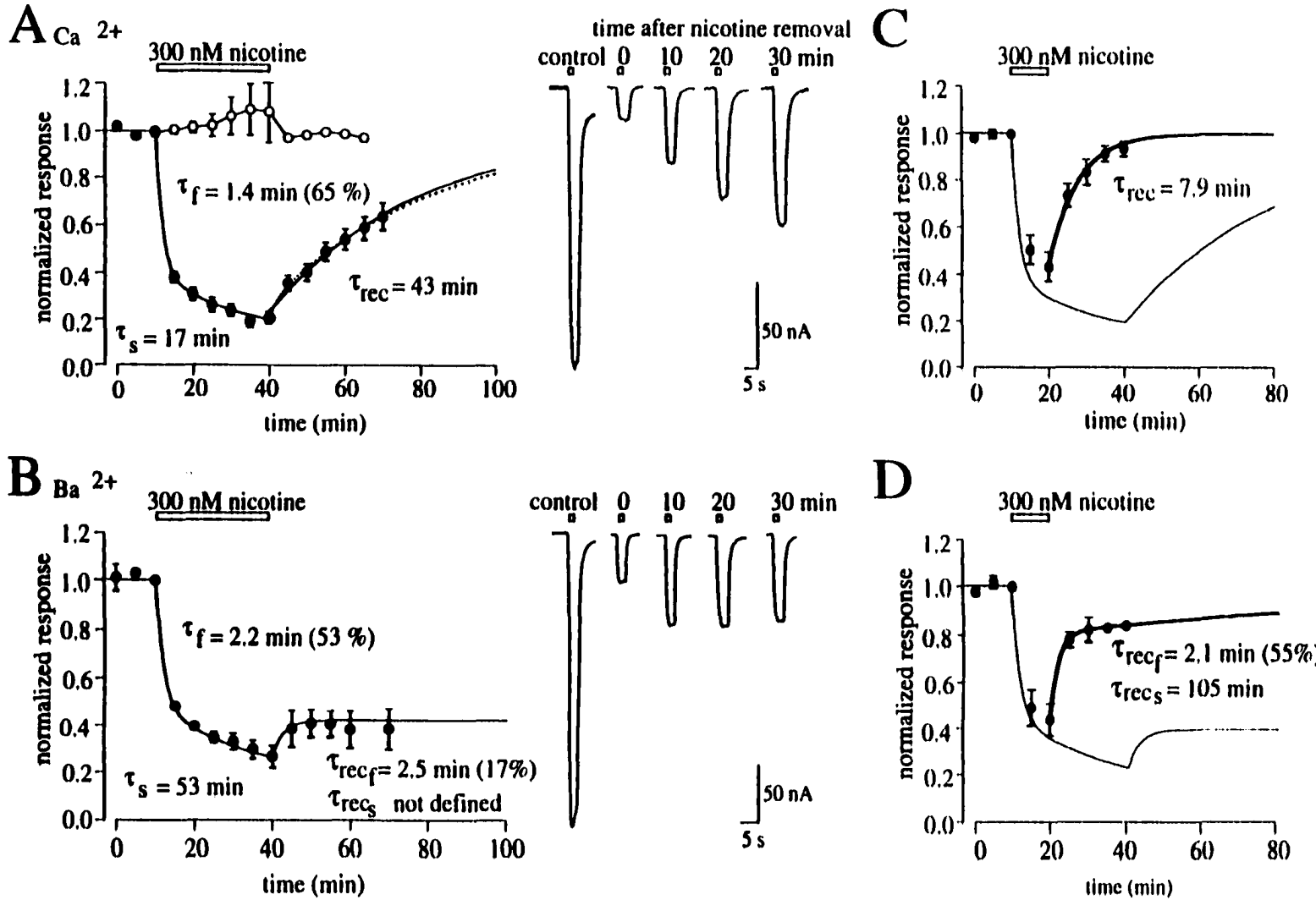
Ca^{2-} Regulates Recovery from Desensitization

It has been shown previously that desensitization of α4β2 receptors induced by 3 min applications of 10 μM nicotine occurs in a biexponential manner, with similar time

constants in the presence or absence of Ca^{2+} (Fenster et al., 1997). However, recovery from desensitization showed a marked dependence on the presence of extracellular Ca^{2+} (Fenster et al., 1997). To further investigate the role of Ca^{2+} on desensitization of $\alpha4\beta2$ nAChRs, experiments were performed after extracellular Ca^{2+} was replaced by Ba^{2+} (Fig. 1).

After obtaining at least three stable test responses to nicotine (10–20 μM; 2 - 10 sec) applied at 5 min intervals, 300 nM nicotine was continuously superfused for a period of 30 min. Test pulse amplitudes were measured and plotted with respect to time before, during, and after the prolonged exposure to nicotine. In the presence of Ca^{2+} (Fig. 1*A*), exponential fits to the peak responses showed that the onset of desensitization was biexponential with fast (τ_f = 1.4 min) and slow (τ_s = 17 min) time constants (n = 18). Mean recovery from desensitization was well described by a single exponential function (τ_{rec} = 43 min). The fractional desensitization at the end of the 30-min exposure to nicotine was 0.79. Although all cells demonstrated recovery from desensitization, there was some variability, particularly between different batches of oocytes (e.g., compare recovery under control conditions in Fig. 2 and 3). Single exponential fits to the recovery phase from individual cells in Ca^{2+} produced values of τ_{rec} that ranged from 16.5 to 97.8 min (mean ± SEM; 42.7 ± 6.8 min, n = 14). Variability of this nature may be expected if recovery from desensitization is regulated by intracellular biochemical processes (see below). To reduce variability, we have, wherever possible, compared oocytes from the same batch. To assess the role of Ca^{2+} on the desensitization process, these results were compared with those from experiments in which Ba^{2+} had been substituted for extracellular Ca^{2+}. During exposure to 300 nM nicotine, the onset and magnitude of desensitization were largely unaffected by Ba^{2+}. Ba^{2+} induced a slowing of the second component of desensitization onset ($p < 0.05$; Fig. 1*B*). However, apart from a small (17%) rapid phase (τ_{rec} = 2.5 min), there was little recovery of $\alpha4\beta2$ nAChRs from desensitization in the presence of Ba^{2+}. These data are consistent with the suggestion that

Figure 1. Recovery from desensitization is reduced in the presence of Ba^{2+}. The time courses of desensitization and recovery were assessed from the inhibition of responses to a repetitively (5 min intervals) applied brief (5-10 sec) test pulse of nicotine (10 μM) during incubations with 300 nM nicotine in the presence of extracellular Ca^{2+} (*A*) or Ba^{2+} (*B*). Time course plots of the test pulse amplitudes during 30 min applications of nicotine (*left*) and example responses from these experiments (*right*) are shown. Data are normalized to control nicotine responses prior to nicotine application. The *solid lines* show double exponential fits to the onset of desensitization, and the fast (τ_f) and slow (τ_s) time constants are indicated. Recovery from desensitization in the presence of Ca^{2+} was fit to both a single exponential (*solid line*) and a double exponential with a fast recovery time constant constrained to 2.5 min (*dashed line*). In the latter fit, the fast component represented 13% recovery and the slow recovery time constant was 54 min. Recovery from desensitization in the presence of Ba^{2+} was fit to a double exponential. The fast time constant represented 17% recovery. The slow time constant could not be defined. The *open circles* in (*A, left*) indicate the stability of the test pulse amplitude in the absence of continuous exposure to nicotine. The effects of a 10 min application of 300 nM nicotine on the rates of recovery from desensitization (*thick lines*) are shown for both Ca^{2+} (*C*; $n = 4$) and Ba^{2+} (*D*; $n = 3$). Recovery was well described by a single exponential component in Ca^{2+} but not in Ba^{2+}. The fitted exponentials for onset and recovery for the 30-min applications are shown superimposed for comparison (*thin lines*).



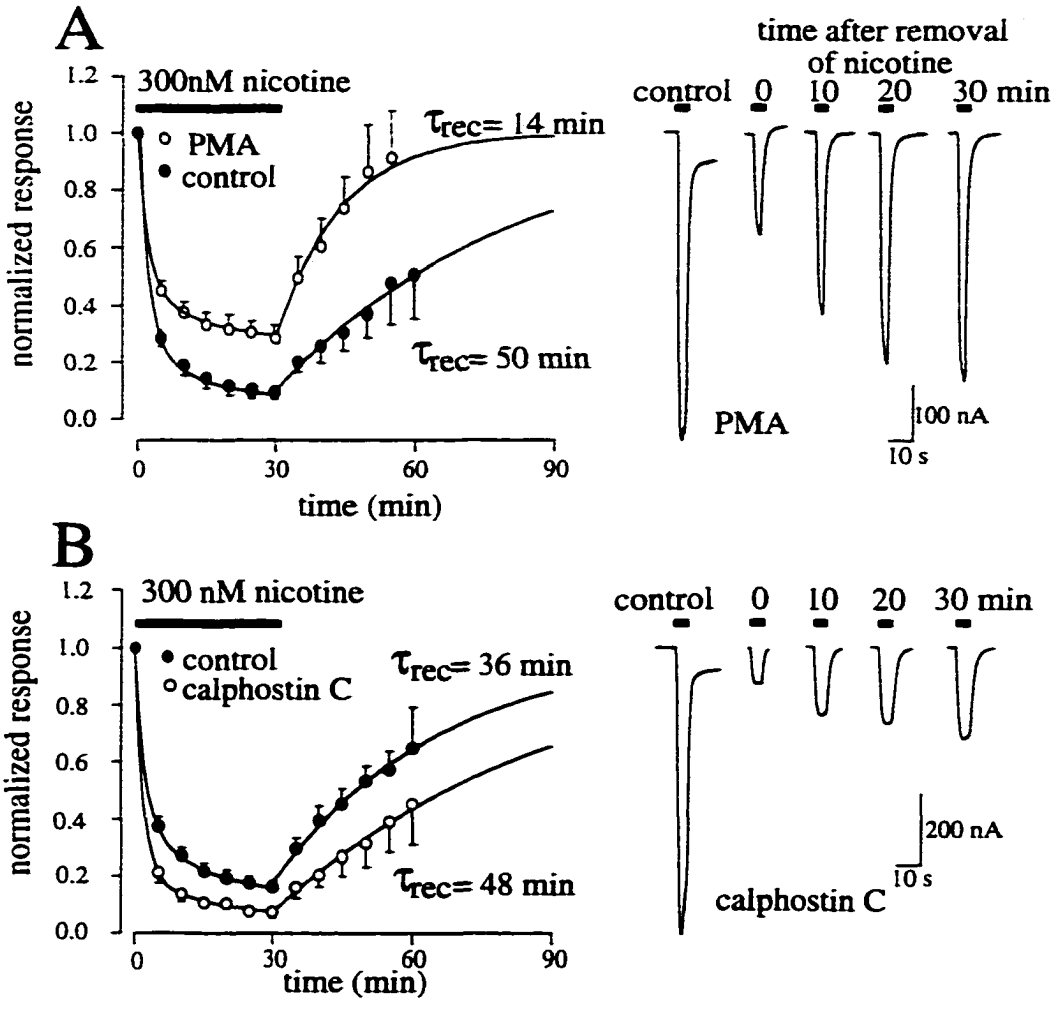


Figure 2. PKC activity regulates the rate of recovery from desensitization. The time courses of desensitization and recovery during a 30 min incubation with 300 nM nicotine in α4β2-expressing oocytes injected with PMA (*A*) or calphostin C (*B*). In each case, these oocytes were compared with controls (uninjected/vehicle-injected) from the same oocyte batch. Time course plots of the test pulse amplitudes (*left*) and example responses (*right*) are shown. Data are normalized to control nicotine responses prior to nicotine application. The *solid lines* show double exponential fits to the onset of desensitization with fast (τf) and slow (τs) time constants for *A*: control, 1.2 (63%) and 9 min (n = 4); PMA, 2.4 (59 %) and 90 min (*n* = 7); and for *B*: control, 2.3 (64%) and 65 min (*n* = 4); calphostin C, 1.8 (82%) and 22 min (*n* = 4). In all cases, recovery from desensitization was well described by a single exponential and the time constant is indicated.

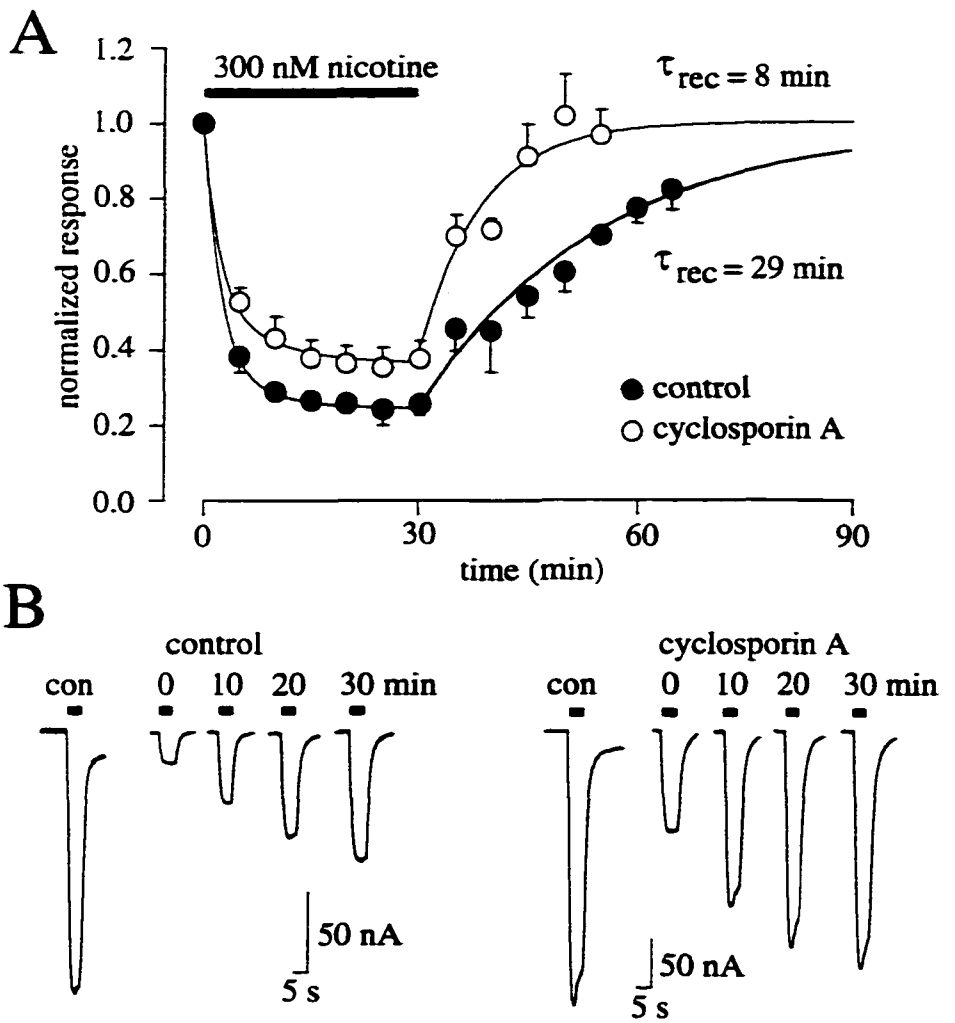


Figure 3. Phosphatase inhibition increases the rate of recovery from desensitization. The time courses of desensitization and recovery during a 30 min incubation with 300 nM nicotine in α4β2-expressing oocytes injected with cyclosporin A. These oocytes were compared with controls (uninjected / vehicle-injected) from the same oocyte batch. Time course plots of the test pulse amplitudes (*A*) and example responses (*B*) are shown. Data are normalized to control nicotine responses prior to nicotine application. The *solid lines* show double exponential fits to the onset of desensitization with fast (τf) and slow (τs) time constants for control of 2.7 (88%) and 22 min ($n = 3$) and for cyclosporin A of 2.2 (76%) and 11 min ($n = 3$). The time courses of recovery from desensitization were in both cases well described by a single exponential and the time constant is indicated.

Ca^{2+} may in part facilitate recovery from desensitization via a Ba^{2+}-insensitive process. Moreover, the data in Ba^{2+} indicate that recovery from desensitization may occur in a biphasic manner. In control experiments (+ Ca^{2+}), the fast component of recovery could have been missed because of its relatively small amplitude; a constrained double exponential fit (with the fast τ_{rec} set to 2.5 min) demonstrated that the fast component would account for 13% of the recovery in the presence of Ca^{2+} (Fig. 1*A*, *dashed line*).

In addition, based on the comparison of recovery from desensitization for the two divalents, we would argue that Ba^{2+} selectively eliminates the slow component of recovery. However, because the fast component of recovery from desensitization is small (≈ 20 %) after 30 min nicotine treatment, it is difficult to accurately assess the effects of Ba^{2+} on this phase of recovery. To address this problem, the time allowed for development of desensitization was limited by reducing the application of 300 nM nicotine to 10 min (Fig. 1*C*, *D*). The rationale is based upon receptor models that have two sequential desensitized states associated with fast and slow transitions, respectively (see below; Feltz and Trautmann, 1982; Boyd, 1989). Because access to the two states is dependent on the time of exposure to agonist, shorter applications will result in fewer receptors in the slowly formed state than in the faster reached state. When agonist is removed, a greater percentage of receptors will recover with a faster time course. As predicted under these conditions, recovery from desensitization became faster in Ca^{2+} (Fig. 1C). More importantly, the relative fraction of the fast phase of recovery was increased in the presence of Ba^{2+}, whereas the slow phase was again essentially absent (Fig. 1*D*). Although we cannot accurately resolve the fast component of recovery from desensitization in Ca^{2+}, this phase appears unaffected by Ba^{2+} since the first 5 min of recovery in Ba^{2+} and Ca^{2+} are essentially the same (compare Fig. 1*C* and *D*). Thus, the effects of Ba^{2+} on desensitization are largely restricted to the slow phase of recovery.

Protein Kinase C Regulates α4β2 Receptor Desensitization

Several mechanisms could account for the enhancement of recovery from desensitization by Ca^{2+}. Two possibilities are that, in addition to enhancing activation (Mulle et al., 1992b; Vernino et al., 1992; Galzi et al., 1996), Ca^{2+} binding to an external site on α4β2 nAChRs also alters desensitization or that Ca^{2+} entry into the oocyte regulates the desensitization recovery process. To address the possible intracellular consequences of Ca^{2+}, we assessed the effects of inhibition and activation of Ca^{2+}-dependent protein kinases and phosphatases. For both muscle-type nAChRs (Hardwick and Parsons, 1996) and neuronal nAChRs in chromaffin cells (Khiroug et al., 1998), recovery from desensitization is dependent upon the state of phosphorylation and is controlled via a Ca^{2+}-dependent phosphatase and PKC. Similar regulation of α4β2 nAChRs is possible, because α4β2 receptors are potential targets for modification by PKC (Goldman et al., 1987; Deneris et al., 1988). Injection of α4β2-expressing oocytes with PMA had two major effects on desensitization (Fig. 2*A*): Compared with untreated oocytes from the same batch of cells, in the presence of extracellular Ca^{2+}, there was an increase in the rate of recovery from desensitization (τ_{rec} = 14 min; $n = 6$; $p < 0.05$) and there was a reduction in the magnitude of desensitization (0.71; $n = 10$; $p < 0.05$). In another batch of oocytes, inhibition of PKC by calphostin C had the opposite effects (Fig. 2*B*). There was an increase in the magnitude of desensitization (0.92; $n = 3$; $p < 0.05$) and the rate of recovery from desensitization was slowed (τ_{rec} = 48 min; $n = 3$; $p < 0.05$). These data imply that PKC activity modulates α4β2 receptor desensitization: activation of PKC enhances recovery from desensitization and its inhibition reduces recovery.

Phosphatase Inhibition Enhances the Rate of Recovery from Desensitization

The PMA and calphostin C data are consistent with the hypothesis that factors that promote phosphorylation enhance the rate of recovery from desensitization. An alternative method of promoting phosphorylation is to suppress dephosphorylation

alternative method of promoting phosphorylation is to suppress dephosphorylation through phosphatase inhibition. Because the function of many ligand-gated channels is affected by the Ca^{2+}-dependent phosphatase calcineurin (Yakel, 1997), the effects of the phosphatase inhibitor cyclosporin A on recovery from desensitization of α4β2 nAChRs were examined. Compared with a control group of oocytes from the same batch, the action of cyclosporin A was almost identical with the effects of activation of PMA (Fig. 3); that is, a dramatic increase in the rate of recovery from desensitization (τ_{rec} = 8 min; n = 3; $p < 0.05$) and a slight decrease in the magnitude of desensitization (0.62; n = 3; $p >$ 0.05). These data support the suggestion that both the extent of α4β2 receptor desensitization and the rate of recovery are determined by the balance of phosphatase and kinase activity.

Elimination of a PKC Phosphorylation Site in the α4 Subunit Inhibits Recovery from Desensitization

The data above imply that modulation of recovery from desensitization of α4β2-containing nAChRs may involve the Ca^{2+}-dependent activation of PKC. One pathway for generating such specificity would be direct phosphorylation of the α4 or β2 subunit by PKC. In addition to two protein kinase A (PKA) sites, the α4 nAChR contains five consensus sites for PKC phosphorylation on the cytoplasmic loop between transmembrane regions 3 and 4, and the β2 subunit contains one PKC site in this region (Goldman et al., 1987; Deneris et al., 1988). One of these sites on the α4 subunit, serine 336, is analogous to a site (serine 333) on the muscle α subunit that is a likely site of PKC-dependent phosphorylation (Huganir et al., 1984). To test the hypothesis that this site is important for recovery from desensitization, a mutant α4 receptor subunit was created in which serine 336 was replaced with alanine. This subunit, denoted $\alpha4S^{336}A$, was expressed in oocytes along with a wild-type β2 subunit. These mutant receptors formed ion channels that were activated and desensitized by nicotine (10 μM) in a

presence Ca^{2+} yielded an EC_{50} value of 13 μM, also similar to wild-type α4β2 receptors (Fenster et al., 1997). The onset of desensitization (nicotine 10 μM; 2 min) could be described by a single (1/6 cells) or a biexponential decay (5/6 cells), with fast [$\tau_f = 6.9 \pm 1.6$ sec (24%) and slow $\tau_s = 135 \pm 20$ sec] time constants; these data are not significantly different from wild-type receptors [$\tau_f = 5.1 \pm 0.4$ sec (24%); $\tau_s = 109 \pm 22$ sec]. The magnitude of desensitization in α4S^{336}Aβ2 nAChRs, estimated at the end of a 2 min nicotine application, was less than in wild-type α4β2 receptors: 0.46 ± 0.02 ($n = 6$) compared with 0.59 ± 0.02 ($n = 7$; $p < 0.05$).

Recovery from desensitization for wild-type α4β2 receptors was compared with recovery for α4S^{336}Aβ2 receptors. Assuming that the mutation at this site on the α4 subunit interferes with the ability of kinases and phosphatases to modulate recovery from desensitization, then the mutant receptor should show slowed recovery from desensitization. Figure 5 shows that although the mutation did not markedly affect the onset of desensitization, as expected from the brief pulses (Fig. 4*B*), there was a profound loss of recovery from desensitization compared with wild-type receptors expressed in the same batch of oocytes. These results are qualitatively similar to those found with Ba^{2+}. That is, after a small (29%) fast phase of recovery [τ_{rec} (fast) = 7.2 min], the slow phase was almost absent, at least during the time course (> 30 min) of the experiment (Fig. 5). Thus, the mutant receptor reproduces one of the effects of PKC inhibition, a slowing of the recovery process, but does not mimic the increase in the extent of desensitization observed with calphostin C (Fig. 2*B*). These data imply that other factors may be important for regulation of α4β2 receptor desensitization.

If the action of PMA involves direct PKC-mediated phosphorylation at serine 336, rather than being independent of PKC (Nishizaki and Sumikawa, 1995), recovery from desensitization in α4S^{336}Aβ2 receptors should not be enhanced by PMA treatment. In the presence of both PMA and cyclosporin A, the overall extent of recovery from

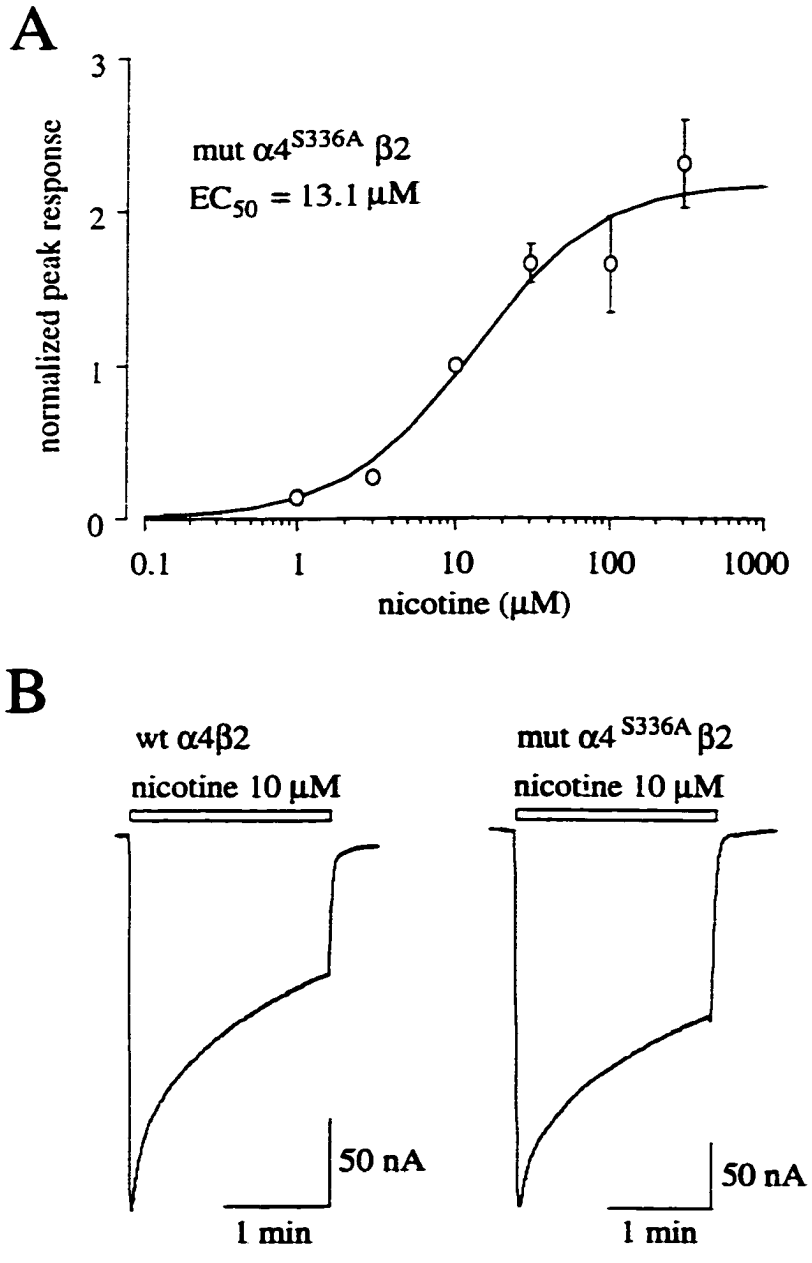


Figure 4. Properties of mutant α4S[336]Aβ2 receptors. *A*, Concentration-response relationship for activation of mutant α4S[336]Aβ2 receptors. The *solid curve* is a logistic fit to the data, with a Hill slope of 1.0. *B*, Comparison of currents induced by 2 min applications of nicotine in wild-type α4β2 (*left*) and α4S[336]Aβ2 (*right*) expressing oocytes.

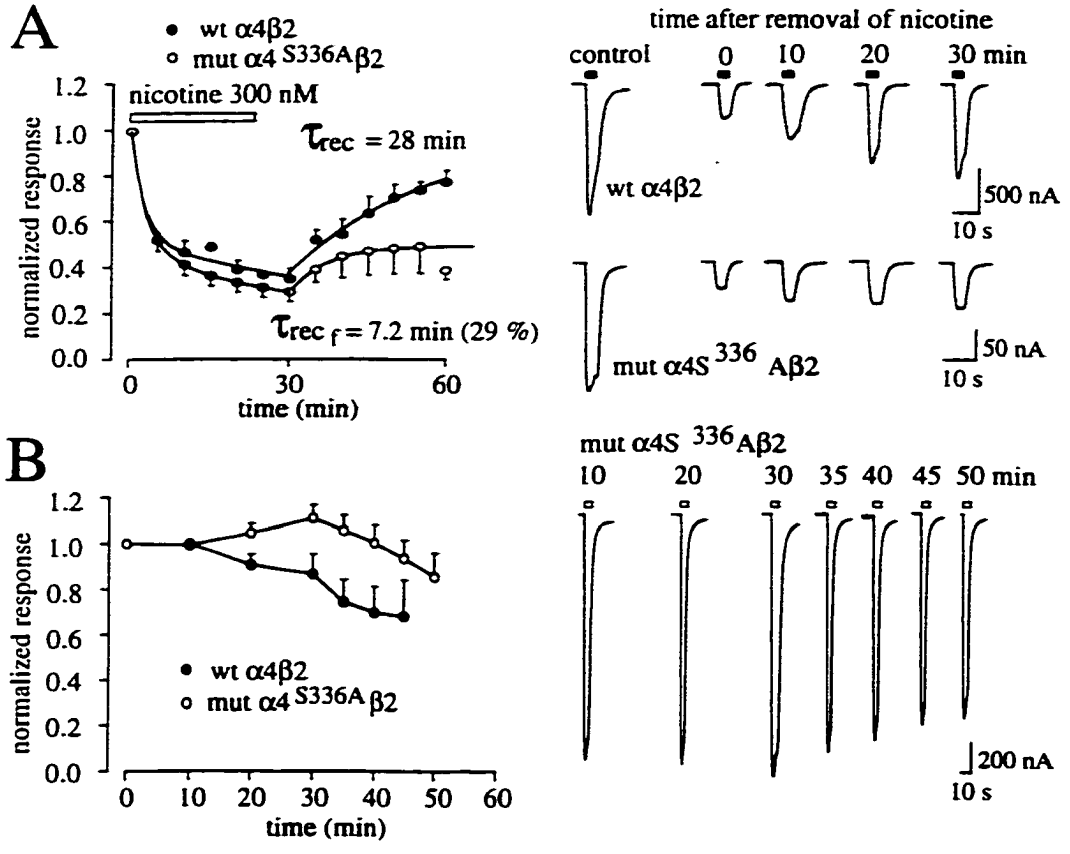
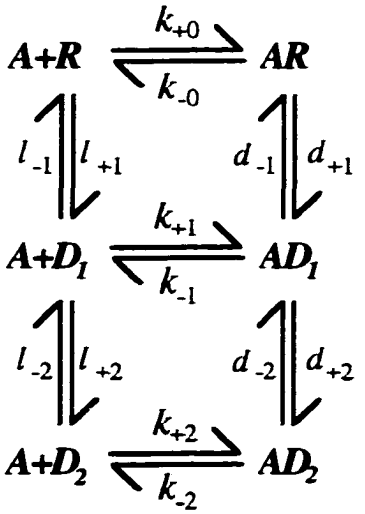


Figure 5. Recovery from desensitization is reduced in mutant α4S ^{336}Aβ2 receptors. *A*, The time courses of desensitization and recovery were assessed during a 30 min incubation with 300 nM nicotine in wild-type α4β2 (*filled symbols*) or mutant α4S^{336} Aβ2 (*open symbol*) receptors. Time course plots of the test pulse amplitudes (*left*) and example responses (*right*) are shown. All oocytes are from the same batch. Data are normalized to control nicotine responses before nicotine application. The *solid lines* show double exponential fits to the onset of desensitization and the fast and slow time constants for wild-type α4β2 and mutant α4S^{336} Aβ2 receptors. Recovery from desensitization of wild-type α4β2 receptors has been fit to a single exponential with its time constant shown. Recovery from desensitization of mutant α4S^{336} Aβ2 receptors was best fit with the sum of two exponentials. *B*, Initial stability of peak amplitudes of test pulses. Brief pulses of nicotine (10 μM) were applied at 5 and 10 min intervals to oocytes expressing either wild-type α4β2 (*filled symbols; n* = 10) or mutant α4S^{336} Aβ2 (*open symbols; n* = 11). Time course plots of the test pulse amplitudes (*left*) and example responses (*right*) are shown.

desensitization of mutant nAChRs was enhanced, as judged from the appearance of a slow phase of desensitization [τ_{rec} (slow) = 63 min; data not shown] that was absent in untreated mutant receptors (see Fig. 5). The fast phase of recovery from desensitization was [τ_{rec} (fast) = 9.0 min]. Because cyclosporin A and PMA did not enhance recovery from desensitization of mutant nAChRs [τ_{rec} (slow) = 63 min] the same extent as wild-type receptors [τ_{rec} = 30 min; data not shown], these results are consistent with the idea that elimination of serine 336 prevents some of the effects of PKC activation on the slow phase of recovery.

Models of α4β2 Receptor Desensitization

The most convenient method for understanding how phosphorylation could regulate α4β2 nAChR function is to examine the consequences of changing transition rates between the various distinct states in a Markov model. As discussed by others, cyclical schemes that incorporate desensitized conformations with high affinities for agonist can describe reasonably well both the onset and recovery of desensitization (Katz and Thesleff, 1957). The biexponential time course kinetics observed in the present study are most readily explained by a model with two desensitized states (Feltz and Trautmann, 1982; Boyd, 1987):



(Scheme 1)

For simplicity, we assume that one molecule of agonist A can produce channel opening by binding to the R state, and that the closed bound state AR is in rapid equilibrium with the open conformation. Binding of agonist to the desensitized states, D_1 and D_2 will cause a loss of receptors in the R states and consequent desensitization. As others have argued previously (Feltz and Trautmann, 1982), we suggest that the rates of formation of AD_1 and AD_2 underlie the fast and slow onset components of desensitization, respectively. Conversely, following removal of agonist, both these two states will unbind agonist relatively rapidly (see below) and the fast and slow recovery phases will be limited by the rates of the transitions from D_1 -> R and D_2 -> D_1, respectively. $K_0 = k_{-0} / k_{+0}$ is the apparent affinity for the activatable state and $K_1 = k_{-1} / k_{+1}$ and $K_2 = k_{-2} / k_{+2}$ are the apparent affinities for the two desensitized conformations. L_1 and L_2 are the allosteric constants describing the ratios of desensitized and activatable receptors, $L_1 = D_1 / R = l_{+1} / l_{-1}$ and $L_2 = D_2 / D_1 = l_{+2} / l_{-2}$. We have shown that the apparent affinity (K_0) of nicotine for rat $\alpha4\beta2$ nAChRs expressed in oocytes is 10 μM (Fenster et al., 1997), which means that the fraction of activatable receptors AR occupied by 300 nM nicotine is very low (≈0.03). Therefore, because ≈ 60% of receptors are "instantaneously" (<2 min) desensitized (Table 1), the fast component of desensitization must proceed via the transition R -> D_1. At very low agonist concentrations, and ignoring the "deep" desensitized state for now, the fraction of activatable receptors in this model is given by (see Feltz and Trautmann, 1982):

$$\frac{R}{R_{max}} = \frac{1 + L_1}{1 + L_1(1 + [A]/K_1)} \qquad (1)$$

At 300 nM nicotine, $R / R_{max} \approx 0.4$ (Fig. 1). If K_1 = 100 nM (a high enough affinity for interaction with nanomolar concentrations of nicotine), then $L_1 \approx 1$. The individual rates

Table 1. Model rate constants

Rate constant (min^{-1})	Control	$\alpha4^{S336A}\beta2$ (Fig. 7*A*)	PMA (Fig. 7*D*)	Calphostin C (Fig. 7*E*)
k_{-1}	500 μM^{-1}			
k_{-0}	5000			
k_{+1}	500 μM^{-1}			
k_{-1}	50			
k_{+2}	500 μM^{-1}			
k_{-2}	0.5			
d_{+1}	24			
d_{-1}	0.24			
d_{+2}	0.064		0.032	0.128
d_{-2}	0.0032	0.00107		
l_{+1}	0.5			
l_{-1}	0.5			
l_{+2}	0.006			
l_{-2}	0.03	0.01	0.06	0.015

Except where changes from control rate constants are shown, all rates remained unaltered.

l_{+1} and l_{-1} can be calculated based on the observation that the equilibration of R and D_1 is fast [i.e. ,$\tau_f \approx 1$ min (Table 1)]:

$$\tau_f = \frac{1}{l_{+1} + l_{-1}} \tag{2}$$

In this case $l_{+1} = l_{-1} = 0.5$ min^{-1}. In this model, the rate constants d_{+1} and d_{-1} will set the time course of the fast phase of desensitization at high agonist concentrations (Dilger and Liu, 1992; see Fig. 7*F*). The time constant τ_f for this process ($\approx$ 5 sec; see Fig. 4) is related to the rate constants by:

$$\tau_f = \frac{1}{f.d_{+1} + d_{-1}} \tag{3}$$

At 10 μM nicotine, f, the fraction of receptors in AR, is 0.5. In addition, because the ratio d_{+1}/d_{-1} is constrained by microscopic reversibility:

$$\frac{d_{+1}}{d_{-1}} = L_1 \frac{K_0}{K_1} \quad (4)$$

and therefore $d_{-1} / d_{-1} = 100$. Substitution of this ratio back into Equation 3 gives $d_{+1} = 24$ min^{-1}, and $d_{-1} = 0.24$ min^{-1}. After the initial fast phase of desensitization, most receptors will be in AD_1 and the slow component of desensitization will reflect the transition AD_1 <-> AD_2. Because, only 10 to 20% recovery occurs with a fast time course, the forward rate constant (and hence the d_{+2} / d_{-2} ratio) must be large enough to drive most receptors into the slowly recovering AD_2 state by the end of a 30 min exposure to nicotine. With a d_{+2} / d_{-2} ratio of 20 and a slow desensitization onset time constant, $\tau_s = 15$ min, $d_{+2} = 0.064$ min^{-1}, and $d_{-2} = 0.0032$ min^{-1}. Provided that equilibrium [^{3}H] nicotine binding reflects the equilibration with the high affinity desensitized state D_2, then a K_2 of 1 nM is consistent with reported values (Wonnacott, 1987). Under these conditions, the second allosteric constant L_2 (l_{+2}/l_{-2}) is also constrained by microscopic reversibility to be 0.2 (see Equation 4). The individual allosteric rates that determine the slow rate of recovery from desensitization can be calculated from Equation 2 assuming a slow recovery time constant, $\tau_{rec} = 30$ min. Again assuming that [^{3}H] nicotine is associated with D_2, k_{-2} should reflect the time course of agonist dissociation. The rate constant for this process has been estimated previously from rat brain membranes as ≈ 0.5 min^{-1} at room temperature (Marks and Collins, 1982). For an affinity constant of 1 nM, the association rate would then be 500 min^{-1}. The final values of all the rate constants are shown in Table 1.

Simulations with Scheme 1 show that pulse durations of 5 to 10 sec could be readily sustained at 5 min intervals after a small loss of response caused by some equilibration of agonist with the deep desensitized state AD_2. This type of use-dependent initial response stabilization (Fig. 6*A*) was often observed in the oocyte experiments (see Fig. 5*B*). A 30-min exposure of the model to 300 nM nicotine produced a biphasic onset

and recovery from desensitization with appropriate time constants (Fig. 6*B*). After 20-min applications of various concentrations of nicotine (Fig. 6*C*; *arrow*), a pseudo-steady-state desensitization dose-response curve was constructed (Fig. 6*D*). The estimated half-maximally effective concentration (IC_{50}) of nicotine for inducing desensitization was 61 nM, similar to values obtained experimentally (Fenster et al., 1997).

The major effect of biochemical manipulation in the present work is consistent with an altered rate of recovery from desensitization. Both Ba^{2+} substitution (Fig. 2) and the mutant receptor (Fig. 5) produced an increase in the time constant associated with the slow phase of recovery, with little apparent effect on the relative amplitudes of the fast and slow components. The time course of the slow phase of recovery is determined by *both* rate constants that govern the transition $D_2 <-> D_1$. However, because the rate out of desensitization l_{-2} is fivefold faster than l_{+2} it will dominate the overall rate of recovery (see Equation. 2). Indeed, with the present model, manipulation of this rate constant was the only method of mimicking the data we observed with the mutant channel. A threefold reduction in l_{-2} produced a slowing of the slow recovery phase with little or no effect on any other process (Fig. 7*A*; Table 1). Thus, we would argue that the effects of the α4 mutation and the actions of Ba^{2+} can be largely explained by slowing the transition D_2 -> D_1. Because these are cyclical schemes, alteration of one rate will destroy microscopic reversibility. To overcome this problem the rate constant d_{-2} for the transition AD_2 -> AD_1 was also changed by the same amount. In addition to making thermodynamic sense, changing both rates that govern return from the "deep" desensitized state seems appropriate because changing the phosphorylation state of the receptor may be expected to have similar effects whether or not agonist is bound (Boyd, 1987; Eilers et al., 1997). A final consequence of altering l_{-2} is to affect the allosteric constant L_2, which will result in a shift in the relative fractions of receptors in the unbound states; a threefold reduction in this rate constant effectively increases the number of desensitized receptors D_2 and consequently reduces the number of activatable receptors R at rest. Thus, an additional

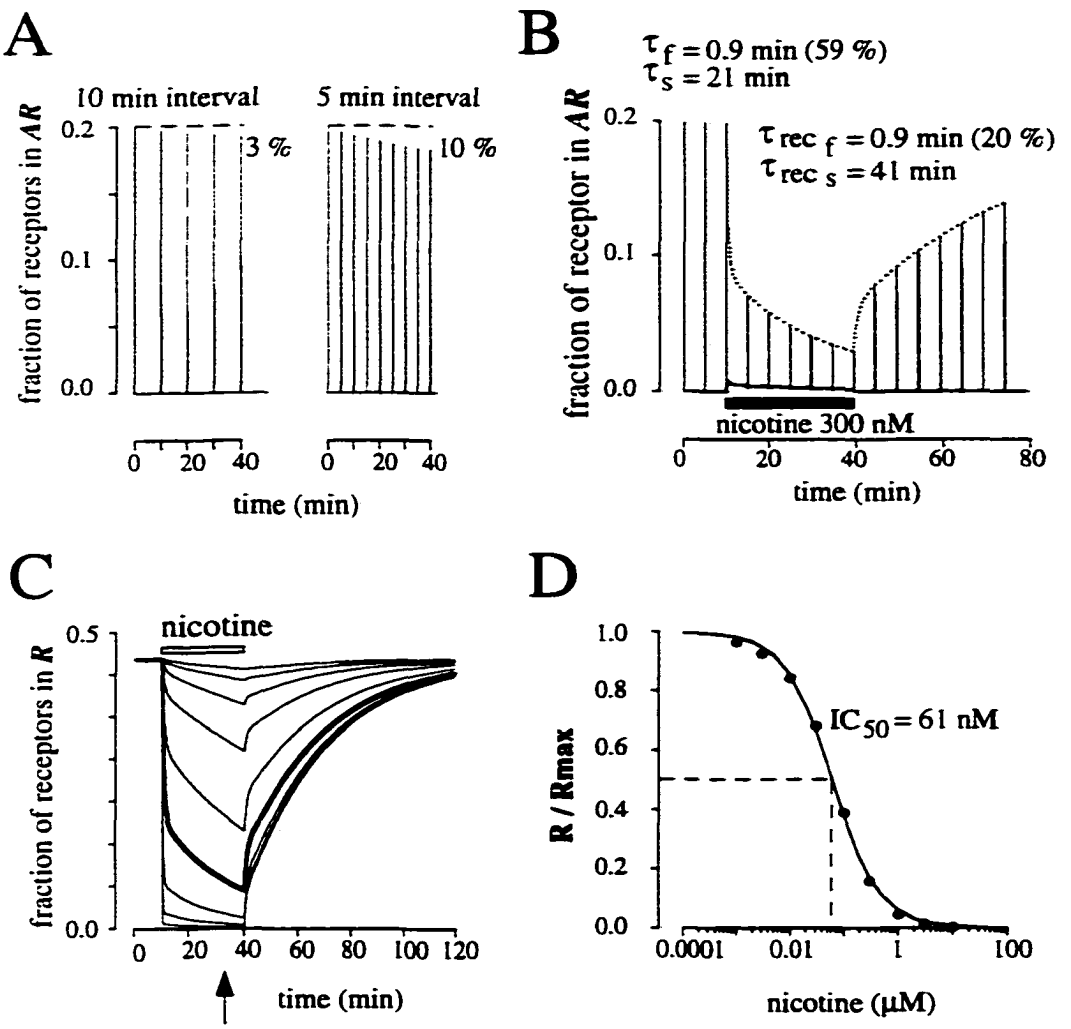


Figure 6. Simulation of α4β2 receptor desensitization. *A*, Initial stability of test pulse amplitude. Simulated responses to 10 sec applications of nicotine (10 μM) at 10 min (*left*) or 5 min (*right*) intervals. The percentage loss of response is shown in each case after 20 min of simulation. *B*, Simulated desensitization experiment using 2 sec pulses of nicotine (10 μM) applied at 5 min intervals before, during, and after 300 nM nicotine for 30 min. Desensitization onset and recovery are described by double exponentials (*dashed lines*) with time constants (and relative amplitudes) indicated. Test pulse reponses were allowed to stabilize before simulated exposure of the model to 300 nM nicotine. *C*, Time course of the change in the fraction of activatable receptors during exposure to various concentrations of nicotine. *D*, Plot of the available receptor fraction after 20 min nicotine exposure (*arrow* in *C*) with respect to the nicotine concentration. The *solid line* is a logistic fit to the simulated data.

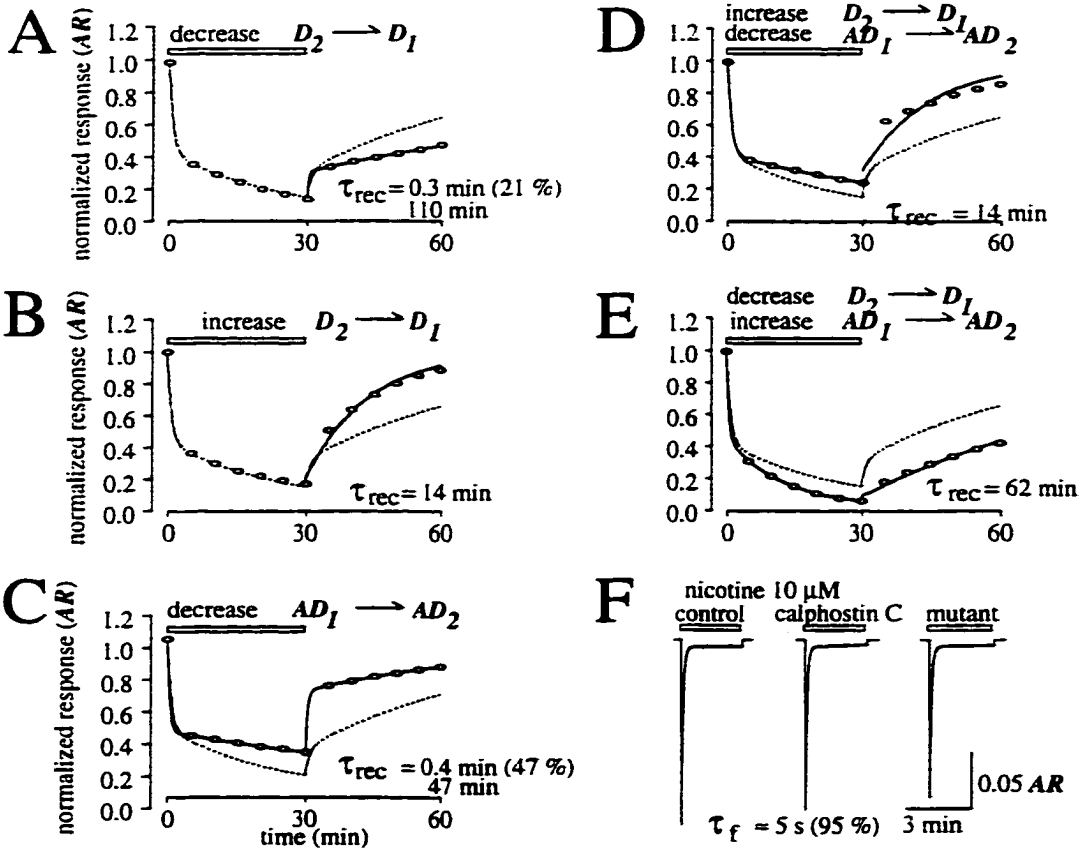


Figure 7. The effects of rate-constant changes on the time course of desensitization. *A*, Simulated test pulse (nicotine, 2 sec; 10 μM) amplitudes during and after 30 min simulated exposure to 300 nM nicotine. The rate of recovery from desensitization is slowed by a threefold reduction in *l*-2 (*d*-2 was reduced by a similar amount to maintain microscopic reversibility). A threefold increase in *l*-2 (and *d*-2) increased the rate of recovery (*B*), whereas a threefold decrease in *d*+2 (and *l*+2) increased the fraction of receptors that recovered with a fast time constant and reduced the magnitude of desensitization (*C*). Opposite rate changes mimic the effects of PKC activation (*D*) and inhibition (*E*) (see Fig. 2). *D*, A twofold increase in *l*-2 balanced by a twofold decrease in *d*+2 enhanced the rate of recovery and decreased the magnitude of desensitization. *E*, A twofold decrease in *l*-2 balanced by a twofold increase in *d*+2 reduced the rate of recovery and increased the magnitude of desensitization. *Open symbols* indicate the test pulse amplitudes and *solid lines* are exponential fits. In *A-E*, the control behavior is shown with dashed lines. F, Simulated currents in response to brief pulses of nicotine (3 min; 10 μM). From left to right, *traces* show responses with control rate constants and adjusted rate constants as in part (*E*; calphostin C) and (*A*; mutant). The fast desensitization time constant and its relative percentage are the same for each of the 3 simulations.

effect of the mutation/dephosphorylation is to slightly reduce the maximal response that can be generated (see Fig. 7*F*).

The effects of the phosphorylation state on desensitization of wild-type nAChRs are more complex than the relatively simple changes associated with the mutant channel. In addition to effects on the slow phase of recovery, there are changes in the magnitude of desensitization. For example, PMA enhances the rate of recovery from desensitization, an effect that can be mimicked by an increase in the rate constant l_{-2}, thereby speeding up the transition D_2 -> D_1 (Fig. 7*B*). However, this manipulation alone does not predict the PMA- and cyclosporin A-induced changes in the magnitude of desensitization (Fig. 2*A*). Slowing the rate constant d_{+2} that controls the onset of desensitization AD_1 -> AD_2 (and l_{+2}; D_1 -> D_2) does decrease the extent of desensitization; however, with this rate change, recovery from desensitization is enhanced by increasing the relative contribution of the fast phase of recovery and not by altering the rate of recovery (Fig. 7*C*). These results suggest that the effects of PKC perturbation may be explained by alterations in both the forward and reverse rate constants that define the transitions between the "shallow" D_1 and "deep" D_2 desensitized states. Attempts to mimic both PKC activation and inhibition are shown in Figure 7, *D* and *E*, respectively. In the case of PMA, the rate of recovery (D_2 -> D_1) is enhanced by a twofold increase in l_{-2} and the magnitude of desensitization (AD_1 -> AD_2) is reduced by a twofold decrease in d_{+2} (note that these two changes are consistent with detailed balancing). The opposite changes in these rate constants produce a desensitization time course that is consistent with inhibition of PKC. Because an increase in the rate of desensitization onset drives more receptors into the "deep" desensitized state by the end of the 30-min nicotine application, the fast component of recovery is largely absent compared with the mutant receptor. This behavior agrees well with the experimental data (Fig. 2*B* and Fig. 5*A*).

DISCUSSION

Protein phosphorylation is essential for G protein-coupled receptor desensitization (Freedman and Lefkowitz, 1996). In the case of ligand-gated channels, phosphorylation plays a modulatory rather than a necessary role in receptor desensitization (Huganir and Greengard, 1990). Examination of the functional properties of $\alpha4\beta2$ nAChRs expressed in *Xenopus* oocytes has revealed that recovery from desensitization is specifically amenable to certain forms of biochemical regulation. It is proposed that Ca^{2+} and factors that promote phosphorylation, possibly directly of the $\alpha4$ subunit, enhance the overall rate of recovery from desensitization.

Desensitization of $\alpha4\beta2$ Receptors

Since the initial studies of Katz and Thesleff (1957), much effort has been invested in defining the process of desensitization in both molecular and biophysical terms. Current models of desensitization for most ligand-gated ion channels are based around cyclical schemes with two distinct desensitized states (Sakmann et al., 1980; Feltz and Trautmann, 1982; Boyd, 1987). Consistent with these studies, we have shown that the time courses of desensitization onset and recovery display biexponential kinetics. It is possible that this type of behavior can be explained by the existence of two separate channels: one with fast and one with slow desensitization properties (e.g., Maconochie and Knight, 1990). However, after short applications of nicotine (10 min), the fast component dominates both the onset of and recovery from desensitization, whereas after 30 min exposure to nicotine, the slow component ($\approx$ 80%) predominates during recovery. This implies that the fast and slow phases are not independent. These data are most readily explained by the existence of a single receptor type with complex desensitization characteristics (Feltz and Trautmann, 1982). Following brief desensitization, most receptors have time only to reach a fast desensitized state, from which recovery is also fast. During prolonged desensitization, more receptors are converted to a slowly reached

desensitized state, from which recovery is also slow. Our analysis of desensitization measures the fraction of receptors that remain in the activatable *R* state during exposure to agonist. Desensitization is effectively the reduction in the relative abundance of this receptor conformation. In the model (see Scheme 1), all the properties of desensitization (e.g. time course, concentration-dependence) are constrained by the rate constants that determine the equilibrium between the various states. Because the fast phase of α4β2 receptor desensitization occurs rapidly (<2 min) and contributes >60% of desensitization onset at 300 nM nicotine, ≈ 50% of channels must exist (in the absence of agonist) in a relatively high affinity "shallow" desensitized state D_1. Agonist rapidly combines with this state to form AD_1 and receptors are rapidly recruited from *R* to D_1 to restore equilibrium. After removal of agonist, the AD_1 state is short-lived (i.e., "shallow") because the large rate constants necessary to permit the rapid onset of desensitization onset *R* -> D_1 also determine its rate of recovery. Thus recovery from desensitization will be fast with brief agonist applications, and slowed after longer periods of agonist, as more receptors have time to accumulate in the longer-lived "deep" desensitized AD_2 conformation (Feltz and Trautmann, 1982; Boyd, 1987). We estimate that ≈ 80% of α4β2 channels can get to this state after a 30-min exposure to 300 nM nicotine. Recovery from this state is slow and is the rate-limiting process of restoring receptor function after prolonged agonist applications.

Regulation of Recovery from Desensitization.

The involvement of phosphorylation in recovery from desensitization of neuronal nAChRs has recently been demonstrated (Khiroug et al., 1998). Boyd (1987), however, first suggested that a biochemical process may specifically regulate the slow phase of neuronal nAChR desensitization. In the present study, we have confirmed this latter idea. We find that Ca^{2+} and PKC are involved in the regulation of the slow phase of recovery from desensitization. For example, with Ba^{2+} substitution, α4β2 receptors displayed a

marked reduction in the slow rate of recovery, with no other differences from control conditions. Mechanistically speaking, this can only be explained by a reduction in the rate constant that allows escape from the "deep" desensitized state $D_2 -> D_1$. The slower rate of recovery from desensitization in Ba^{2+}-containing media implies that Ca^{2+} may be important for normal α4β2 receptor function. It seems unlikely that the action of Ca^{2+} results from direct binding to an extracellular site on the receptor (Mulle et al., 1992b; Vernino et al., 1992; Galzi et al., 1996) because Ba^{2+}, which can replace Ca^{2+} in its ability to increase nAChR responses (Mulle et al., 1992b), does not substitute for Ca^{2+} in the enhancement of recovery from desensitization. Therefore, Ca^{2+} influx, perhaps in part via the nAChR channel itself (Mulle et al., 1992a; Vernino et al., 1992), may be important for its effects on recovery. Ca^{2+} could be acting directly on the intracellular face of the receptor (Miledi, 1980; Cachelin and Colquhoun, 1989) or through activation of various Ca^{2+}-dependent kinases and phosphatases, as suggested for nAChRs in chromaffin cells (Khiroug et al., 1998). We have shown that activation and inhibition of PKC enhances and attenuates the slow rate of recovery from desensitization of α4β2 nAChRs, respectively, consistent with the idea that factors that promote phosphorylation facilitate recovery from the "deep" desensitized state. Because recovery from desensitization can be enhanced both by phosphatase inhibition and by PKC activation, it is suggested that the rate of recovery from desensitization will be governed by the relative balance of kinase and phosphatase activity. Because the effects of Ba^{2+} in wild-type α4β2 receptors are almost identical to the behavior of the mutant $\alpha4S^{336}A\beta2$ nAChR, we would argue that the dominant role of Ca^{2+} under the present conditions is to facilitate recovery from desensitization, possibly through activation of PKC.

α4 Subunits Are Potential Substrates for PKC

Direct phosphorylation of α4 nAChR subunits by PKA (Nakayama et al., 1993) is enhanced by chronic treatment with nicotine (Hsu et al., 1997). Although various muscle

nAChR subunits act as PKC substrates (Huganir and Greengard, 1990), no equivalent direct PKC-mediated phosphorylation of neuronal nAChRs has been demonstrated. As predicted from our results with inhibition and activation of PKC, the PKC-site mutant $\alpha4S^{336}A\beta2$ nAChR exhibited a decrease in the slow time constant of recovery from desensitization, which suggests that PKC-dependent phosphorylation at this site may be important for recovery of function. However, the effects of inhibition of PKC were not restricted entirely to the recovery from desensitization; there was also an increase in the magnitude of desensitization. Based on our model, we suggest that this may be explained by an increase in the rate constant governing entry into the "deep" desensitized state. In other reports, the onsets of desensitization of muscle-type nAChRs (Huganir et al., 1986; Hoffman et al., 1984) and nAChRs in sympathetic ganglia (Downing and Role, 1987) are enhanced by PKA and PKC activation, respectively. In the case of chromaffin cells, any action of PKC on desensitization onset and/or steady-state response may have been missed (Khiroug et al., 1998), because the high concentrations of agonist used would rapidly drive the majority of receptors into the "shallow" desensitized state and the transition to the "deep" state would become largely silent (see Fig. 7*F*). Furthermore, because the rate of recovery from desensitization in the mutant receptor could be partially enhanced by a combination of cyclosporin A and PMA, it is likely that the effects of phosphorylation on desensitization are not limited to one site on α4 subunits. Indeed the α4 subunit alone contains 5 potential PKC sites (Goldman et al., 1987) and can be heavily phosphorylated in oocytes *in vivo* (Viseshakul et al., 1998). Our results do not necessarily indicate that PKC directly phosphorylates this site; rather, the mutation could be affecting the interaction of the α4 subunit with some intermediate protein or it could confer a conformational change in the subunit that itself alters the rate of recovery from desensitization. In this respect, we did observe a slight decrease in the magnitude of desensitization in mutant nAChRs that was not predicted.

Overall, although the kinetic model and its rate constants are not likely to be unique solutions for α4β2 receptor desensitization, together with the experimental data, these results imply that there is more than one site of action of PKC on α4β2 nAChRs, and that the effects of phosphorylation and dephosphorylation are probably confined to the transitions between the "deep" and "shallow" desensitized states. There is no direct evidence phosphorylation can regulate native α4β2 nAChRs in neurons; however, the desensitization properties of nAChRs both in chromaffin cells (Khiroug et al., 1998) and at the neuromuscular junction (Hardwick and Parsons, 1996) are modulated by phosphorylation, as are those for N-methyl-D-aspartate (Tong et al., 1995) and γ-aminobutyric-acid$_A$ (Martina et al., 1996) receptors. These data imply that phosphorylation-dependent regulation of desensitization may be a general mechanism for ligand-gated ion channels (Huganir and Greengard, 1990).

Implications for Nicotine Addiction

Prolonged exposure to levels of nicotine related to use of tobacco upregulates the number of high-affinity (α4β2) nicotine binding sites in the CNS (Marks et al., 1983; Schwartz and Kellar, 1985; Flores et al., 1992) and in heterologous expression systems (Peng et al., 1994; Gopalakrishnan et al., 1996). In contrast to the increase in receptor number, the functional responsiveness of nAChRs is markedly reduced (Lukas, 1991; Marks et al., 1993; Peng et al., 1994). It has been suggested that reduced function is a consequence of nAChRs entering a "permanently inactive" state (Lukas, 1991; Peng et al., 1994), probably via agonist-induced desensitized conformations (Boyd, 1987). Previously we have demonstrated that α4β2 nAChRs have an intrinsically slow rate of recovery from desensitization after a 30 - 60 min treatment with levels of nicotine related to use of tobacco (Fenster et al., 1997). We suggest here that desensitization of nAChRs induced by prolonged exposure to nicotine may result in a reduced Ca^{2+} influx, thereby promoting the dephosphorylated state of α4β2 receptors. Recovery from the "deep"

desensitized conformation would be markedly slowed, and receptors would become "trapped" in a chronically desensitized/deactivated state (Lukas, 1991; Peng et al., 1994). Indeed, it has been reported that prolonged treatment with PKC inhibitors will also drive $\alpha4\beta2$ nAChRs to a functionally inactive conformation (Eilers et al., 1997). Chronic PMA treatment, which downregulates PKC activity (Favaron et al., 1990), promotes an increase in the number of $\alpha4\beta2$ receptors (Gopalakrishnan et al., 1997), consistent with the suggestion that the dephosphorylated state of the receptor could serve as either directly or indirectly a signal for preventing receptor turnover (Peng et al., 1994).

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SUMMARY AND CONCLUSIONS

Summary of Results

A systematic characterization of the concentration ranges over which nicotine activates and desensitizes different nAChR subtypes should reveal which receptor subtypes are "targets" of nicotine at levels obtained from tobacco. Therefore, in the first study, titled "*Influence of Subunit Composition on Neuronal Acetylcholine Receptors at Low Concentrations of Nicotine*" (Fenster et al., 1997), nicotine-induced activation and desensitization of different nAChR subtypes was investigated for several nAChR subtypes expressed in *Xenopus* oocytes, specifically, $\alpha3\beta4$, $\alpha3\beta2$, $\alpha4\beta4$, $\alpha3\beta4$, and $\alpha7$ nAChRs. The contribution of individual subunits on receptor function was determined by comparing the functional properties of these different receptor subtypes. Briefly, we find that (1) the $\alpha4$ subunit contributes high relative affinity for nicotine activation and functional desensitization; (2) $\beta2$-containing nAChRs exhibit faster desensitization kinetics then their $\beta4$-containing counterparts, (3) recovery from desensitization is slower for nAChRs with high relative affinities for functional desensitization (i.e., $\alpha4$-containing receptors), and (4) $\alpha7$ homomeric receptors display very rapid desensitization kinetics and low relative affinities for nicotine activation and desensitization.

From dose-response curves constructed for nicotine activation and steady-state desensitization, predictions can be made regarding the outcome of prolonged nicotine exposure on the function of these different nAChR subtypes. For all of the subtypes examined, apparent affinities for nicotine steady-state functional desensitization are orders of magnitude higher than for activation; however, there is a range of concentrations in which the activation and desensitization curves overlap. At these concentrations nicotine can continually produce some nAChR activation because

desensitization is incomplete. For example, the major outcome of prolonged exposure to tobacco-related levels of nicotine (20-500 nM) is to stabilize α4-containing nAChRs in the high-affinity desensitized state. While this reduces the population of α4-containing nAChRs available for activation by endogenous acetylcholine, nicotine at these concentrations will produce continuous low-level activation of α4β2 nAChRs.

In addition to apparent affinities for nicotine activation and steady-state desensitization, we characterized the rates at which these different receptor subtypes desensitized and recovered from nicotine-induced desensitization. We find that desensitization kinetics is both subunit and concentration dependent; for example, desensitization is faster at higher nicotine concentrations and is also faster for nAChRs containing β2 subunits. Rates of recovery, however, are generally slower for α4-containing nAChRs (i.e., receptors of higher affinity), which suggests that recovery rates are governed, in part, by the affinity of the receptor for the desensitized state. For example, α4β4 nAChR function did not completely recover even after two hours of washout following prolonged (60 min) nicotine exposure. In contrast to these slow rates of recovery, α3β2 nAChRs fully recovered within a few minutes of washout. From these studies that investigate desensitization and recovery from desensitization, predictions can be made regarding the rates at which brain nAChRs are desensitized as nicotine levels rise (as occurs during tobacco use) and the rates at which they recover as nicotine levels begin to fall (as occurs after tobacco use). One such prediction is that α3β2-containing brain nAChRs will recover from nicotine-induced desensitization more rapidly than α4β2-containing nAChRs.

Physiologically relevant changes in extracellular Ca^{2+} levels have been shown to influence the sensitivity of neuronal nAChRs to agonist activation (Mulle et al., 1992a, b; Lena and Changeux, 1997). Therefore, we compared the functional properties of the different nAChR subtypes in the presence of extracellular Ca^{2+} and in the "absence" of extracellular Ca^{2+}. For α7 homomeric receptors, apparent affinities for activation and

desensitization are shifted to the left (i.e., higher affinity) in the presence of extracellular Ca^{2+}. The affinities of the other nAChRs tested were significantly affected by the presence of absence of external Ca^{2+}. The effects of external Ca^{2+} on desensitization and recovery kinetics were also examined. Rates of recovery from desensitization for α4β2 nAChRs, the major high-affinity brain nAChR subtype, are enhanced by the presence of extracellular Ca^{2+}. Extracellular Ca^{2+} did not significantly influence the other receptor subtypes. Recovery from desensitization has been shown to be modulated by Ca^{2+}-dependent second messengers for muscle-type nAChRs (Hardwick and Parsons, 1996) and neuronal type nAChRs (Khiroug et al., 1997, 1998). Our results suggest that a similar mechanism may modulate rates of recovery for α4β2 nAChRs.

A conclusion that can be drawn from the results of the first study is that, with regular tobacco use (in which nicotine levels may remain chronically elevated between 10-300 nM nicotine) high-affinity α4β2-containing nAChRs will become stabilized in desensitized state(s). In addition to desensitization, chronic exposure to low (tobacco-related) levels of nicotine causes an upregulation in the number of α4β2–containing nAChRs both in brain and in heterologous expression systems (Flores et al., 1992; Peng et al., 1994a; Bencherif et al., 1995; Whiteaker et al., 1998). Together, these findings are consistent with the hypothesis that a desensitized receptor state serves as a "trigger" for upregulation of nAChR number.

In the second study, titled "*Upregulation of Surface α4β2 Nicotinic Receptors Is Initiated by Receptor Desensitization after Chronic Exposure to Nicotine*"(Fenster et al., 1999b), we provide several lines of evidence which support the "desensitization-hypothesis" for upregulation of surface α4β2 nAChRs expressed in *Xenopus* oocytes. First, we find that the nicotine concentration required for half-maximal upregulation (~10 nM) is equal to the concentration required for both half-maximal functional desensitization and equilibrium binding to surface receptors in intact oocytes. We also find that a mutant α4β2 nAChR that does not readily recover from desensitization

(Fenster et al., 1999b) can be upregulated in the absence of nicotine. In addition, we provide evidence that desensitization also serves as a "trigger" for the upregulation of other nAChR subtypes. Specifically, we find that $\alpha3\beta4$ nAChRs, which require higher nicotine concentrations for functional nAChR desensitization, also require comparably higher concentrations for upregulation. Because different nAChR subtypes exhibit different affinities for nicotine-induced desensitization, our findings, which suggest that desensitized states initiate upregulation of different nAChR subtypes, will be useful for predicting the long-term consequences of chronic nicotine treatment.

In summary, these results support the idea that receptor desensitization and upregulation are closely related phenomena. In contrast, previous studies have concluded that the "desensitization-hypothesis" for upregulation is false because the concentrations of agonist required for upregulation are orders of magnitude higher than estimates of concentrations required for desensitization (Peng et al., 1994a; Bencherif et al., 1995; Whiteaker et al., 1998). However, their estimates of the agonist concentrations required for desensitization were obtained indirectly from equilibrium [^{3}H]agonist binding assays performed on membrane homogenates, rather than directly by functional desensitization or from binding to cell intact preparations. We find that the EC_{50} value for equilibrium [^{3}H]nicotine binding to $\alpha4\beta2$ nAChRs from membrane homogenates (~ 0.4 nM) is orders of magnitude less than the EC_{50} values (~10nM) obtained for both functional desensitization or equilibrium [^{3}H]nicotine binding to surface $\alpha4\beta2$ nAChRs on intact cells. These results suggest that equilibrium binding to receptors from membrane homogenates differs from both functional desensitization and equilibrium binding of surface receptors on intact cells. This difference may be caused by the existence of internal receptors with binding affinities that differ from surface receptors (Wonnacott, 1987). For example, we find that, compared with total equilibrium [^{3}H]nicotine binding to receptors on intact oocytes, total equilibrium binding is quantitatively greater (sixfold) after membrane homogenization. This suggests that approximately 80% of total nAChRs

are intracellular. However, the existence of intracellular receptors does not exclude the possibility that membrane homogenization, through biochemical changes, shifts both intracellular and surface nAChRs to higher affinity states. In summary, it appears that the estimates of the affinity for the desensitized state of surface receptors cannot be accurately measured from equilibrium [^{3}H]agonist binding assays. This may account for the discrepancies between apparent affinities for equilibrium binding and upregulation observed previously in other systems (Peng et al., 1994a; Whiteaker et al., 1998).

If desensitization and upregulation of α4β2 nAChR function are related phenomena, as our results suggest, then factors that modulate α4β2 nAChR desensitization may influence not only changes in receptor function but also changes in receptor number that occur with prolonged exposure to nicotine at tobacco-related levels. In the third study, titled "*Regulation of α4β2 Nicotinic Receptor Desensitization by Calcium and Protein Kinase C*" (Fenster et al., 1999a), we demonstrate that α4β2 nAChR desensitization is strongly influenced by physiologically relevant factors, specifically extracellular Ca^{2+} and PKC.

As mentioned above, we provide evidence in the first article that, for α4β2 nAChRs expressed in *Xenopus* oocytes, recovery from desensitization is dependent upon the presence of extracellular Ca^{2+}. These findings lend some support the to idea that Ca^{2+} influx through α4β2 nAChRs may activate Ca^{2+}-dependent intracellular second messengers that in turn modulate α4β2 nAChR function. A comparison of desensitization in the presence of extracellular Ca^{2+} and in the presence of Ba^{2+} (instead of Ca^{2+}), reveals that the fast component of desensitization is largely unaffected by either condition; However, in the presence of Ba^{2+} there is a large reduction in the second (i.e., slow) component of desensitization. In addition, there is almost no recovery from desensitization apart from a small rapid phase in the presence of Ba^{2+}. These results suggest (1) that desensitization is a biphasic process and (2) that Ca^{2+} may enhance recovery from a "deep" desensitized state (i.e., the state revealed by the slow component)

through a Ba^{2+} insensitive process. Because extracellular Ba^{2+} does not substitute for Ca^{2+}, it is unlikely that the effects of Ca^{2+} on α4β2 nAChR desensitization are the result of a direct interaction of Ca^{2+} with binding sites on the receptor (Mulle et al., 1992b; Galzi et al., 1996).

To examine the possibility that activation of a Ca^{2+}-dependent PKC underlies the effects of extracellular Ca^{2+} on α4β2 nAChR desensitization, desensitization and recovery from desensitization were examined after treatment with PMA (an activator of PKC) and calphostin-C (an inhibitor of PKC). Treatment with PMA both reduces the magnitude of desensitization and enhances rates of recovery from desensitization. Treatment with calphostin-C had the opposite effects, both increasing the magnitude of desensitization and reducing rates of recovery from desensitization. In summary, these results demonstrate that activation of PKC enhances recovery from desensitization and that PKC inhibition reduces recovery from desensitization.

To investigate the possibility that PKC-dependent modulation of α4β2 nAChR desensitization is mediated through changes in the phosphorylation state of the receptor, we examined desensitization and recovery from desensitization after treatment with cyclosporin, an inhibitor of the Ca^{2+}-dependent phophatase calcineurin. It is predicted that treatment with cyclosporin-A will increase the phosphorylation-state of the receptor by inhibiting receptor dephosphorylation. Treatment with cyclosporin-A, similar to treatment with PMA, reduces magnitude of desensitization and dramatically enhances rates of recovery from desensitization. Because both PKC activation and phosphatase inhibition should promote receptor phosphorylation, these data are consistent with the idea that the balance of phosphatase and PKC activity influences α4β2 desensitization and recovery after desensitization.

To investigate further the possibility that direct phosphorylation of the receptor underlies the apparent influence of PKC activity on α4β2 nAChR desensitization, we also examined desensitization and its recovery for a mutant α4β2 nAChR that lacks a

PKC consensus site on the α4 subunit ($\alpha4S^{336}A\beta2$). As observed following replacement of extracellular Ca^{2+} with Ba^{2+}, recovery from desensitization, apart from a small fast phase, is inhibited for the mutant α4β2 nAChR. It is predicted that if the effect of PKC activation (by PMA) exclusively involves direct phosphorylation at this site, PMA and/or cyclosporin-A treatment should not influence recovery from desensitization for the mutant $\alpha4S^{336}A\beta2$ nAChR. However, combined treatment of the mutant $\alpha4S^{336}A\beta2$ nAChR with PMA and cyclosporin-A results in the appearance of a slow phase of recovery that is absent for untreated mutant $\alpha4S^{336}A\beta2$ nAChRs. In summary, these results are consistent with the idea that elimination of serine 336 prevents some of the effects of PKC activation on the slow phase of recovery from desensitization.

Rates of desensitization and recovery observed for α4β2 nAChRs following treatment with activator and inhibitors of PKC, and for the mutant α4β2 nAChR were examined in the context of Markov model. The biphasic kinetics of desensitization, and the differences between rates of desensitization and recovery, can be explained most simply by a cyclical model in which there are two desensitized states (Feltz and Trautmann, 1982; Boyd, 1987). In the context of this model (see Fig. 3), extracellular Ca^{2+} and/or PKC-dependent receptor phosphorylation appear to enhance rates of return from the "deep" desensitized state (i.e., revealed by the slow component of desensitization) to the "shallow" desensitized state (i.e., revealed by the fast component of desensitization).

Discussion

Activation and desensitization of nAChRs. We and others (Leutje and Patrick, 1991; Cachelin and Rust, 1995; Chavez-Noriega et al., 1997; Stauderman et al., 1998) report that individual nAChR subunits influence overall receptor sensitivity to nicotine-induced activation and desensitization. For example, it has been demonstrated that α4-

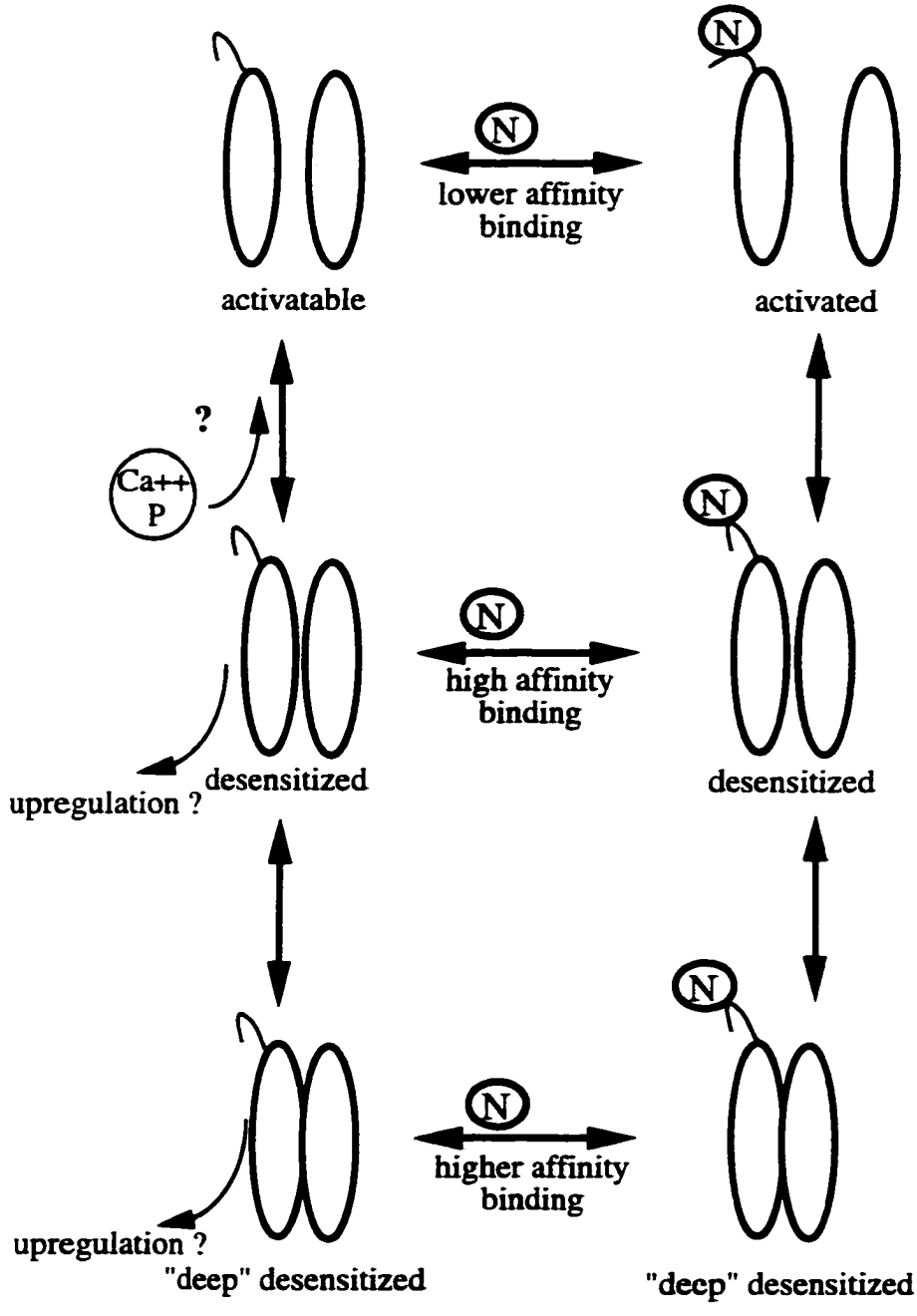

Figure 3. Cyclical model of desensitization.

containing nAChRs exhibit high relative affinities for nicotine activation and desensitization (Hussy et al., 1994; Peng et al., 1994a; Gopalakrishnan et al., 1996), which implies that α4-containing receptors are the primary "targets" for nicotine at levels obtained from tobacco. Different nAChR subtypes also exhibit different apparent affinities for acetylcholine-induced activation (Chavez-Noriega et al., 1997; Stauderman et al., 1998), which may be relevant to nicotinic activity in brain. For example, the distance of nAChRs from sites of acetylcholine release is often difficult to establish because of an apparent lack, in many cases, of direct synaptic nicotinic transmission in brain (Colquhoun and Patrick, 1997). Therefore, it is possible that those nAChR subtypes most sensitive to acetylcholine are targets for activation by acetylcholine that has diffused from neighboring sites of release. Because nAChRs exhibit higher affinities for agonist-induced desensitization, it is possible that extremely low levels of acetylcholine that diffuse from distant sites of release desensitize some nAChRs. In addition, we find that the sensitivity of α7 nAChRs to agonist-induced activation/desensitization is enhanced in the presence of extracellular Ca^{2+}. This has been attributed to an allosteric interaction of Ca^{2+} with external sites on the α7 subunit that results in an increase in channel open probability (Mulle et al., 1992b; Galzi et al., 1996). This suggests a mechanism in which physiologically relevant reductions in extracellular Ca^{++}, such as occurs with increased/sustained synaptic activity, cause a reduction in total α7 nAChR activity (i.e., reduced opening probability) but prevent α7 nAChR desensitization (i.e., reduced affinity for functional desensitization). This might serve to prevent total rundown of the nicotinic response and to preserve sustained low level activity.

Receptor desensitization is a highly conserved feature among the family of ligand-gated ion channels; however, we are just beginning to appreciate the physiological roles of desensitization (Jones and Westbrook, 1996). At the neuromuscular junction, the current decays within several milliseconds, whereas desensitization is much slower (tens of milliseconds). Therefore, it has been difficult to establish a physiological role for

desensitization at the neuromuscular junction (Jones and Westbrook, 1996). In brain, however, the sites of acetylcholine release in relation to nAChRs are largely unknown; thus, the time course and concentrations of acetylcholine that interact with nAChRs in brain remains speculative. It is possible, therefore, that nAChR desensitization plays an important role in shaping synaptic transmission in brain. For example, the activity of α7 and α3β2 nAChRs, which exhibit the most rapid rates in desensitization (McGehee and Role, 1995), might be dramatically reduced with high frequency/sustained stimulation. Desensitization of these nAChRs might also serve as a negative-feedback mechanism to prevent excitotoxicity that could occur with sustained Ca^{2+} entry through activated nAChRs (see Jones and Westbrook, 1996). In summary, brain nAChRs are thought to play an important modulatory role, as indicated by Ca^{2+} permeability sufficient to activate Ca^{2+}-dependent Cl^- and K^+ channels (Seguela et al., 1993), to influence responses by other ligand-gated receptors (Mulle et al., 1992a), and to facilitate the neurotransmitter release (Gray et al., 1996). Therefore, desensitization of these nAChRs may have far-reaching effects on brain neurotransmission.

Rates of recovery from desensitization will, like desensitization, influence the number of activatable nAChRs at highly active synapses. Specifically, nAChRs that recover very rapidly can be repetitively stimulated. For example, we find that α3β2 and α3β4 nAChRs recover more rapidly than their α4-containing counterparts (Hsu et al., 1996). α3-containing nAChRs are highly expressed in parasympathetic ganglia. The rapid rates of recovery of these nAChRs from desensitization may serve a physiological requirement for high-gain / fail-safe nicotinic stimulation at these synapses.

Regulation of nAChR desensitization. Knowledge regarding factors that govern the rates at which nAChRs desensitize and recover from desensitization should lead to an understanding of how neurons control the level of nAChR activity. For α4β2 nAChRs expressed in *Xenopus* oocytes, we find that rates of return to activatable states after

prolonged (≥ 30 min) nicotine exposure is strongly influenced by the balance of PKC and phophatase activity. Consistent with these findings, others have also demonstrated that, for α4β2 nAChRs expressed in HEK cells, recovery from desensitization induced by prolonged nicotine is enhanced by activators of PKC and reduced by inhibitors of PKC (Eilers et al., 1997). Together, these findings suggest that regulation of α4β2 nAChR desensitization is not dependent upon the type of cell in which the receptor is expressed and is likely to occur in neurons. This type of regulatory mechanism implies that, in neurons, nAChR activity could be influenced by increases in intracellular Ca^{++} and/or activation of G protein-coupled receptors. In summary, the regulation of nAChR desensitization and recovery from desensitization by phosphorylation / dephosphorylation may influence both short-term and long-term sensitivity of nAChRs to acetylcholine.

It is possible that regulation of desensitization by phosphorylation / dephosphorylation may control nAChR activity even in the absence of agonist. For example, it has been demonstrated that, in the absence of agonist, downregulation of α4β2 nAChR function occurs in the presence of PKC inhibitors (Eilers et al. 1997). The simplest explanation of these findings is that even in the absence of agonist, a fraction of nAChRs exist in desensitized states at equilibrium (Lippiello et al., 1987; Margiotta et al., 1987). It follows that, if rates of return from desensitized states are inhibited by receptor dephosphorylation, as appears to be the case for α4β2 nAChRs, then a greater fraction of receptors will exist in desensitized states at equilibrium. In addition to downregulation of α4β2 nAChR function, upregulation of α4β2 nAChR number has been observed with PKC inhibition in the absence of agonist (Eilers et al., 1997; Gopalakrishnan et al., 1997). These results are consistent with evidence provided in the second article, which suggested that α4β2 nAChR desensitization serves as a "trigger" for upregulation; therefore, it is possible that PKC activity regulates not only α4β2 nAChR desensitization, but also upregulation of receptor number that occurs with chronic nicotine. Consistent with this possibility, we find that the mutant $\alpha4S^{336}A\beta2$ nAChR (see Chapter 2), which

exhibits reduced recovery from desensitization, is more readily upregulated. These findings imply that biochemical modulation alone may significantly influence both the function and number of nAChRs in neurons.

"Permanent inactivation," desensitization, and upregulation. After chronic nicotine exposure (≥ 1 day), the rates at which α4β2 receptors return to activatable states was extremely slow to negligible (see also Hsu et al., 1996). Recovery of α4β2 receptor function is indeed so slow that receptors have been described as permanently inactivated (Peng et al., 1994a). Although recovery from desensitization induced by less prolonged nicotine exposure (30 min) is relatively slow for α4β2 nAChRs (as demonstrated in the first article), these slow rates of recovery do not account for permanent inactivation of these receptors. Because the length of time that a receptor spends in a desensitized state is governed by the time constant of the rate out of that state, if rates of recovery from a desensitized state are inhibited, receptors may appear to be permanently inactivated. Consistent with this idea, we find that recovery from desensitization is inhibited both for α4β2 nAChR after PKC inhibition and for the mutant α4S^{336}Aβ2 receptor. These experimental manipulations appear to cause permanent inactivation of these receptors. Our data suggest that the effects of both PKC inhibition and the elimination of the PKC consensus site at serine 336 are confined to the "deep" state of desensitization that, for a majority of the receptors, can only be reached after prolonged agonist exposure. It is possible that permanent inactivation of α4β2 nAChRs is a consequence of the accumulation of receptors in the "deep" desensitized state during chronic nicotine exposure and a subsequent biochemical step (involving receptor dephosphorylation) that inhibits recovery from "deep" desensitized states (see Figure 3).

It has been hypothesized that permanent inactivation (rather than desensitization) initiates upregulation of α4β2 nAChR number in *Xenopus* oocytes (Peng et al., 1994a). While we suggest that desensitized states serve as the "trigger" for upregulation, recovery

of both α4β2 and α3β4 nAChR function following removal of chronic nicotine was extremely slow. The almost negligible rates of return to activatable states for these receptors suggest that it might be more accurate to say that these receptors were inactivated (rather than desensitized). Our results, therefore, are consistent with the hypothesis that permanently inactivated states trigger upregulation of receptor number. However, there is evidence that desensitized receptor states might be sufficient to "trigger" upregulation and that permanent inactivation is not necessarily required. For example, some nAChRs (including α4β2 nAChRs in some expression systems) may be upregulated both in number and in function (Rowell and Wannacott, 1990; Golpalikrishnan et al., 1996). This might occur if receptors are upregulated in number and are not inhibited from returning to activatable states. Therefore, we suggest that for surface α4β2 nAChRs expressed in *Xenopus* oocytes, α4β2 nAChR desensitization (and an additional biochemical step involving receptor dephosphorylation) may underlie both permanent inactivation and receptor upregulation.

Madhok and colleagues (1995) provide evidence consistent with the idea that, after desensitization, an additional biochemical step is required for nAChR upregulation. Specifically, a threefold increase in the number of α3-containing nAChRs was observed in wild-type PC-12 cells after chronic nicotine exposure. However, for mutant PC-12 cells deficient in PKA, no nAChR upregulation was observed after chronic nicotine. Interestingly, for wild-type PC-12 cells cAMP levels are increased with prolonged nicotine exposure (Baizer and Weiner, 1985). In addition, cAMP alone can enhance [^{3}H]nicotine binding (i.e., increase nAChR number) in PC-12 cells (Madhok et al., 1995). Therefore, a mechanism is proposed in which PKA-dependent phosphorylation causes (or influences) nAChR upregulation by somehow affecting assembly of receptor subunits or transport of receptors to the cells surface, or by preventing degradation of receptors on the membrane (Madhok et al., 1995).

Is desensitization the trigger for upregulation? The second article, we provide evidence that upregulation of surface α4β2 nAChRs in *Xenopus* oocytes appears to be triggered by desensitization. However, upregulation of intracellular α4β2 nAChRs requires higher concentrations of nicotine (EC_{50} = 60 nM) than are required for equilibrium desensitization of either surface (EC_{50} = 10 nM) or internal nAChRs (EC_{50} = 0.4 nM). This suggests that desensitization does not necessarily trigger upregulation of intracellular α4β2 nAChRs. However, it is possible that measurements of the equilibrium binding affinity of intracellular nAChRs are altered by membrane homogenization. It follows that, if we could measure the equilibrium binding affinity of intracellular α4β2 nAChRs in intact cells, it might correspond to the "affinity" of the intracellular receptors for nicotine-induced upregulation. Another possibility is that upregulation of intracellular α4β2 nAChRs is the result of internalization of surface nAChRs that are not degraded, as has been observed for α3 nAChRs in SH-SY5Y cells (Peng et al., 1997). In this case, desensitization could indirectly trigger upregulation of internal nAChRs through its ability to trigger upregulation of surface nAChRs. In summary, the signal for upregulation of intracellular receptor remains unclear.

We provide evidence that desensitization may also trigger upregulation of other nAChR subtypes. Specifically, we show that functional desensitization and upregulation are induced by similar concentrations of nicotine for α3β4 nAChRs. In contrast, α3β4 nAChRs expressed in human embryonic kidney (HEK) cells are not upregulated by chronic agonist exposure (Wang et al., 1998). However, α3β2 nAChRs expressed in these HEK cells are dramatically (~24-fold) upregulated by chronic nicotine. It is suggested that the inability of α3β4 nAChRs to be upregulated by chronic nicotine is the result of the differential ability of the internal domains of the β2 and β4 subunits to interact with an unknown intracellular protein (Wang et al., 1998). For example, β4 but not β2 subunits (when expressed in place of the β1 subunits of muscle-type nAChRs) can associate with rapsyn, a protein that aggregates and anchors nAChRs to the

cytoskeleton. Because α3β4 nAChRs in these HEK cells appear to both form clusters and to exhibit high surface expression levels relative to α3β2 nAChRs (prior to chronic-nicotine), it is suggested that the β4 subunit interacts with an intracellular protein in HEK cells that stabilizes the receptor on the cell surface. Another possibility is that these HEK cells are deficient in an enzyme (i.e., PKA) required for α3β4 nAChR upregulation (Madhok et al., 1995). In summary, it is also possible that desensitization does not necessarily trigger upregulation of all nAChRs subtypes and/or of nAChRs expressed in different types of cells. While desensitization may trigger an upregulation in the number of some nAChR subtypes, there are clearly other (and as yet unknown) steps involved. Identifying the biochemical steps that influence nAChR upregulation and the cellular mechanisms (i.e., decreased in nAChR degradation, increased nAChR subunit assembly, etc.) that underlie the process of upregulation will need to be addressed in future studies.

Additional directions of future research. Comparisons between nAChRs expressed in *Xenopus* oocytes and HEK cells reveal that, although sensitivities to agonist-induced activation are similar, desensitization is orders of magnitude faster (≥ 100-fold) for nAChRs expressed in HEK cells (Chavez-Noriega et al., 1997; Stauderman et al., 1998; Wang et al., 1998). These results suggest that factors related to the host cell strongly influence nAChR kinetics (Lester and Dani, 1995). In addition, after chronic nicotine, we observe an upregulation in the number of α3β4 nAChRs in oocytes. In contrast, α3β4 nAChRs in HEK cells do not appear to be upregulated even with chronic exposure to high nicotine levels (Wang et al., 1998). Therefore, it will be necessary to expand the results of these studies (in oocytes) to nAChRs expressed in HEK cells and, ultimately, to neurons. Future studies will also be directed toward identifying the cellular factors that underlie differences observed between nAChRs expressed in oocytes and HEK cells.

Nicotine Addiction

Our ability to understand the mechanisms through which nicotine (at tobacco-related levels) produces reward, addiction, and tolerance is complicated by several factors which include the following: (1) A large number of functionally distinct receptor subtypes exist in brain (see McGehee and Role, 1995; Colquhoun and Patrick, 1997); (2) prolonged nicotine exposure can reversibly desensitize different nAChRs subtypes; whereas, longer-term nicotine exposure may cause receptor "inactivation" (Hsu et al., 1996; Fenster et al., 1999); (3) changes in external Ca^{2+} and/or the phosphorylation state of the receptor may influence desensitization and recovery from desensitization Fenster et al., 1999a); and (4) chronic nicotine causes a dramatic increase in the number of some nAChR subtypes. These factors make it difficult to determine the net effects of nicotine on the function of different nAChRs subtypes. The diverse physiological effects of nicotine may be linked to the diverse outcomes that nicotine has on receptor function, which include receptor activation, desensitization, inactivation, and upregulation (Dani and Heinemann, 1996; Lindstrom, 1997). For example, the rewarding effects of nicotine have been linked to activation of nAChRs that enhance dopamine release in the "reward pathway" (Calabresi et al., 1989; Picciotto et al., 1998). Nicotine sensitivity, i.e., the perception of the first cigarette of the day as the most rewarding, has been attributed to recovery from desensitization of receptors that may also be upregulated in number (Dani and Heinemann, 1996; Lindstrom, 1997). Withdrawal symptoms associated with cessation of tobacco use have been associated with the recovery of upregulated nAChRs. This may result in heightened activation of nAChRs by endogenous acetylcholine (Dani and Heineman, 1996). Finally, nicotine tolerance has been associated with receptor desensitization and/or inactivation (Dani and Heinemann, 1996; Lindstrom, 1997). Although we can speculate as to the outcomes of nicotine on nAChR function (and number) and the relationship of these outcomes to the physiological effects of nicotine, there are several fundamental questions that remain unanswered. For example, it remains

to be established whether nicotine exerts its effects directly or by disrupting nAChR activation by endogenous acetylcholine. It also remains to be established which receptor subtypes underlie specific behavioral consequences of nicotine.

Our data are consistent with the following hypothetical model of nicotine addiction (see Fig. 4): The initial elevation of nicotine during tobacco use (1 μM) activates nAChRs that stimulate release of dopamine within the "central reward" mesolimbic system (Calabresi et al., 1989; Picciotto et al., 1998). High-affinity α4β2-containing nAChRs are the primary subtype activated by nicotine at these levels (Fenster et al., 1997; Lindstrom, 1997). Because the half-life of nicotine is about 2 hr, lower levels (10-300 nM) of nicotine remain for some time in the smoker's blood (Benowitz et al., 1989). Because nicotine preferentially interacts with the desensitized state of nAChRs, the primary action of prolonged nicotine exposure is to cause desensitization of some nAChR subtypes. Specifically, a majority of α4-containing receptors are desensitized (Fenster et al., 1997). Other lower affinity (non-α4 containing) receptor subtypes may also be desensitized, but to a lesser extent (Fenster et al., 1997; Lindstrom, 1997). With regular tobacco use, chronic nicotine exposure results in both "permanent inactivation" and upregulation of nAChR number. We suggest that both of these processes are the outcome of nAChR desensitization. A fundamental question regarding nicotine addiction relates to how the nicotine-induced desensitization and upregulation (of receptor number) affects the excitability of nAChRs (Wonnacott, 1990). Our data in oocytes suggests that overall receptor function is reduced, despite dramatic increases in receptor number (Fenster et al., 1999b). This downregulation in receptor function may account for nicotine tolerance (Dani and Heinemann, 1996). After a period of abstinence, desensitized receptors (and possibly inactivated receptors) may begin to return to activatable states. The rates at which nAChRs return to activatable states will depend upon nAChR subtype, the duration and level of nicotine exposure, and the balance of kinase and phosphatase activity (Fenster et al., 1997; 1999a). Because nAChRs are upregulated in number,

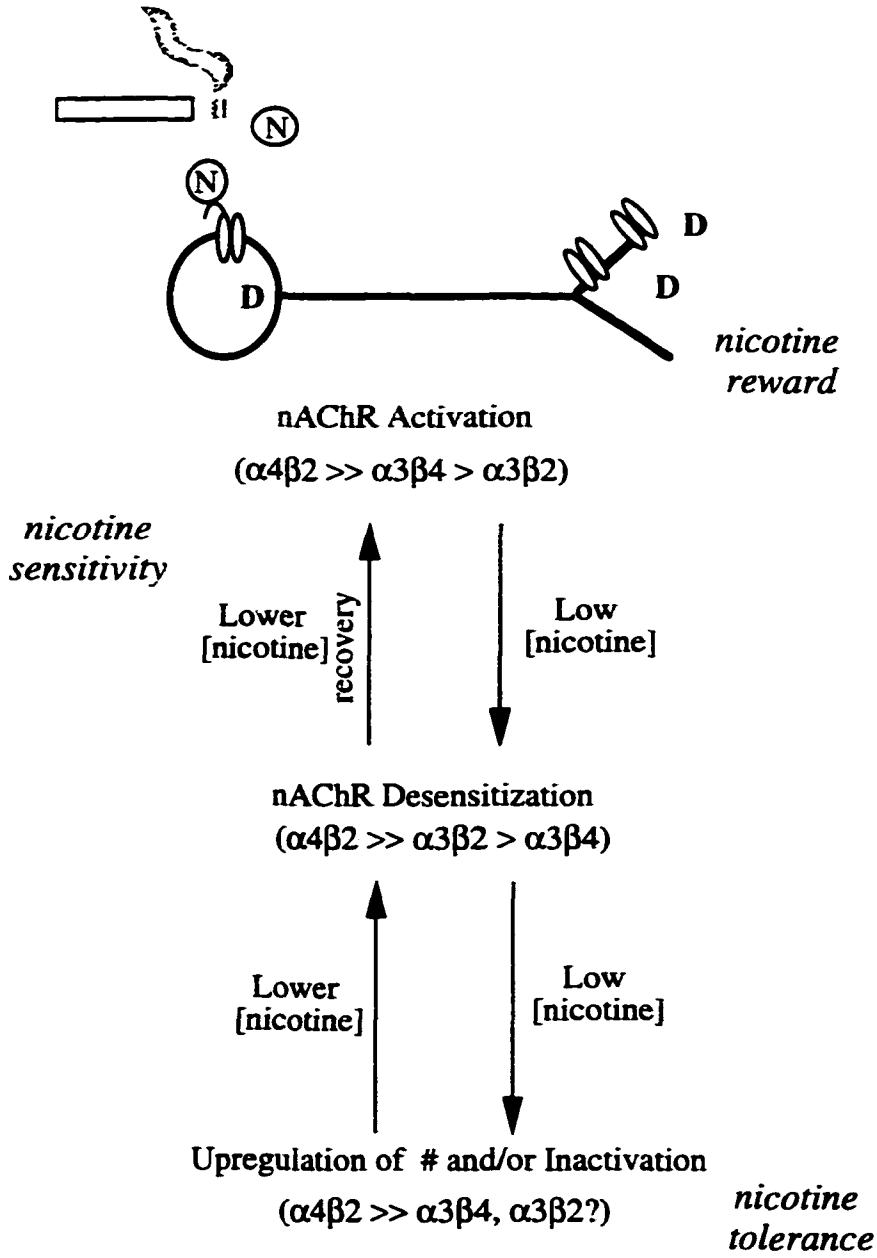

Figure 4. Model of nicotine addiction.

recovery from desensitization and/or inactivation might, in brain, ultimately result in a net increase of nAChR excitability by endogenous acetylcholine (Dani and Heinemann, 1996). Such increases in overall nAChR excitability may underlie, in part, nicotine sensitivity and withdrawal (Dani and Heinemann, 1996).

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM
DOCTOR OF PHILOSOPHY

Name of Candidate Catherine Pichon Fenster

Major Subject Neurobiology

Title of Dissertation Desensitization and Upregulation of Neuronal Nicotinic Acetylcholine Receptors

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name	Signature
Michael W. Quick, Ph.D., Chair	
J. Edwin Blalock, Ph.D.	
Kent T. Keyser, Ph.D.	
Robin A.J. Lester, Ph.D.	
David S. Weiss, Ph.D.	

Director of Graduate Program Craig Garner

Dean, UAB Graduate School Joan F. Lorden

Date 12/9/99