

Regulation of $\alpha 4\beta 2$ Nicotinic Receptor Desensitization by Calcium and Protein Kinase C

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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 $\alpha 4\beta 2$ nAChRs expressed in *Xenopus laevis* oocytes were investigated. Nicotine-induced (300 nM; 30 min) desensitization of $\alpha 4\beta 2$ receptors in the presence of Ca^{2+} developed in a biphasic manner with fast and slow exponential time constants of $\tau_f = 1.4$ min (65% relative amplitude) and $\tau_s = 17$ min, respectively. Recovery from desensitization was reasonably well described by a single exponential with $\tau_{\text{rec}} = 43$ min. Recovery was largely eliminated after replacement of external Ca^{2+} with Ba^{2+} and slowed by calphostin C ($\tau_{\text{rec}} = 48$

min), an inhibitor of PKC. Conversely, the rate of recovery was enhanced by phorbol-12-myristate-13-acetate ($\tau_{\text{rec}} = 14$ min), a PKC activator, or by cyclosporin A (with $\tau_{\text{rec}} = 8$ min), a phosphatase inhibitor. $\alpha 4\beta 2$ receptors containing a mutant $\alpha 4$ 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, $\alpha 4\beta 2$ nAChRs accumulate in a "deep" desensitized state, from which recovery is very slow. We suggest that PKC-dependent phosphorylation of $\alpha 4$ 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 $\alpha 4\beta 2$ receptors observed after chronic nicotine treatment.

The family of neurotransmitter-gated ion channels is responsible for fast synaptic transmission in the peripheral and central nervous systems (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 func-

tion 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 $\alpha 4$ and $\beta 2$ subunits contribute

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ABBREVIATIONS: CNS, central nervous system; nAChR, nicotinic acetylcholine receptor; PKC, protein kinase C; PKA, protein kinase A; PMA, phorbol-12-myristate-13-acetate.

to a majority of the high-affinity nicotine binding sites in the brain (Whiting and Lindstrom, 1988; Flores et al., 1992). Furthermore, nicotine at levels related to tobacco use (Benowitz et al., 1989) both activates and desensitizes $\alpha 4\beta 2$ nAChRs (Hsu et al., 1995; Fenster et al., 1997); desensitization may initiate the up-regulation of nAChR number that occurs during chronic nicotine exposure (Wonnacott, 1990; Schwartz and Kellar, 1985). Therefore, factors that regulate $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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.

Experimental Procedures

Expression of Functional nAChRs in *X. laevis* 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 $\alpha 4$ subunit ($\alpha 4^{\text{S336A}}$) 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 to 96 h 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 M Ω . 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 before experimentation, so as to avoid direct extracellular effects of protein kinase activators/inhibitors on nAChR channels (see 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 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- to 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 of the 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 2-fold 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 s; 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 $(I_{\text{con}} - I_{\text{final}})/I_{\text{con}}$, 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- to 3-min application of 10 μM nicotine. For statistical comparison of mean data, weighted-means Student's *t* tests (for unpaired comparisons) and paired Student's *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 \pm S.E.M. 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 $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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 s) 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. 1A), exponential fits to the peak responses showed that the onset of desensitization was biexponential with fast ($\tau_f = 1.4$ min) and slow ($\tau_s = 17$ min) time constants ($n = 18$). Mean recovery from desensitization was well described by a single exponential function ($\tau_{\text{rec}} = 43$ 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 Figs. 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 \pm S.E.M.; 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 ($P < .05$; Fig. 1B). However, apart from a small (17%) rapid phase ($\tau_{\text{rec}} = 2.5$ min), there was little recovery of $\alpha 4\beta 2$ nAChRs from desensitization in the presence of Ba^{2+} . These data are consistent with the suggestion that 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. 1A, 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 ($\approx 20\%$) 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 and 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, 1987). 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

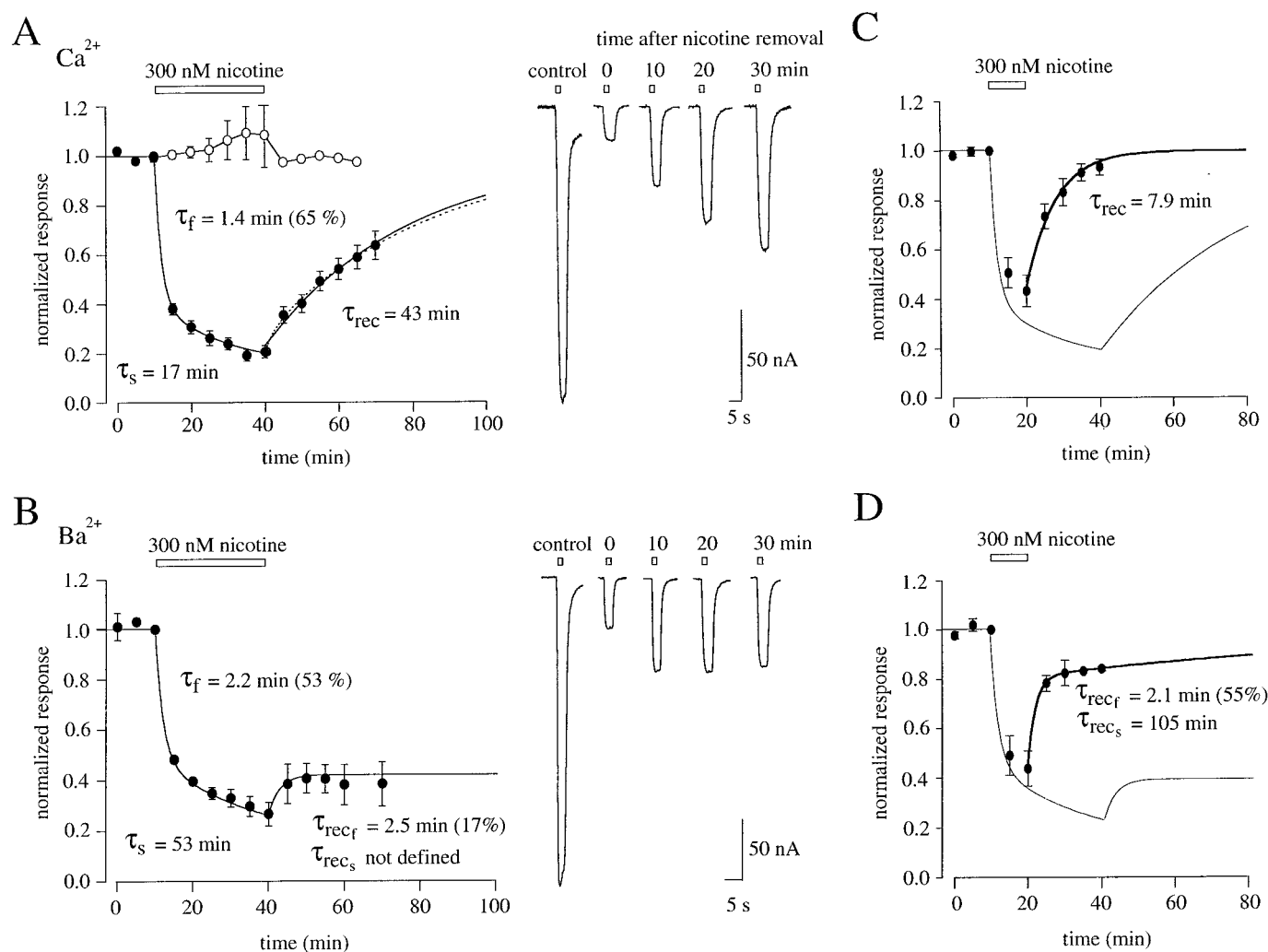


Fig. 1. Recovery from desensitization is reduced in the presence of Ba^{2+} . The time courses of desensitization and recovery were assessed from the inhibition of responses to a repetitively applied (5-min intervals) brief (5–10 s) test pulse of nicotine (10 μM) during incubations with 300 nM nicotine 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 before 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. \circ 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).

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. 1D). Although we cannot accurately resolve the fast component of recovery from desensitization in Ca^{2+} , this phase seems unaffected by Ba^{2+} , because 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.

PKC Regulates $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ nAChRs is possible, because $\alpha 4\beta 2$ receptors are potential

targets for modification by PKC (Goldman et al., 1987; Deneris et al., 1988). Injection of $\alpha 4\beta 2$ -expressing oocytes with PMA had two major effects on desensitization (Fig. 2A): 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 ($\tau_{\text{rec}} = 14$ min; $n = 6$; $P < .05$) and there was a reduction in the magnitude of desensitization (0.71; $n = 10$; $P < .05$). In another batch of oocytes, inhibition of PKC by calphostin C had the opposite effect (Fig. 2B). There was an increase in the magnitude of desensitization (0.92; $n = 3$; $P < .05$) and the rate of recovery from desensitization was slowed ($\tau_{\text{rec}} = 48$ min; $n = 3$; $P < .05$). These data imply that PKC activity modulates $\alpha 4\beta 2$ receptor desensitization: activation of PKC enhances recovery from desensitization and its inhibition reduces recovery.

Phosphatase Inhibition Enhances 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 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 $\alpha 4\beta 2$ nAChRs were examined. Compared with a control group of

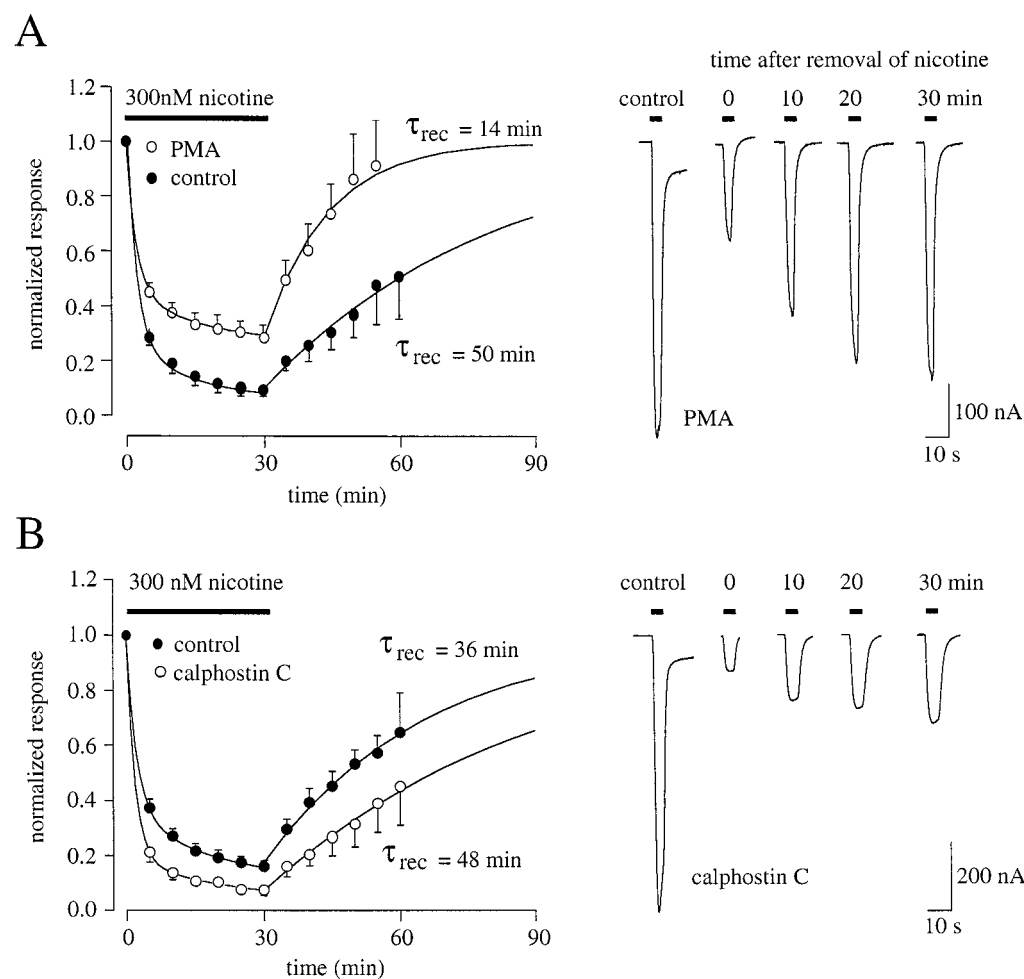


Fig. 2. PKC activity regulates the rate of recovery from desensitization. The time courses of desensitization and recovery during a 30-min incubation with 300 nM nicotine in $\alpha 4\beta 2$ expressing oocytes injected with PMA (A) or calphostin C (B). In each case, these oocytes were compared with control oocytes (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 before nicotine application. The solid lines show double exponential fits to the onset of desensitization with fast (τ_f) and slow (τ_s) 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.

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 ($\tau_{\text{rec}} = 8$ min; $n = 3$; $P < .05$) and a slight decrease in the magnitude of desensitization (0.62 ; $n = 3$; $P > .05$). These data support the suggestion that both the extent of $\alpha 4\beta 2$ receptor desensitization and the rate of recovery are determined by the balance of phosphatase and kinase activity.

Elimination of a PKC Phosphorylation Site in $\alpha 4$ Subunit Inhibits Recovery from Desensitization. The data above imply that modulation of recovery from desensitization of $\alpha 4\beta 2$ -containing nAChRs may involve the Ca^{2+} -dependent activation of PKC. One pathway for generating such specificity would be direct phosphorylation of the $\alpha 4$ or $\beta 2$ subunit by PKC. In addition to two protein kinase A

(PKA) sites, the $\alpha 4$ nAChR contains five consensus sites for PKC phosphorylation on the cytoplasmic loop between transmembrane regions 3 and 4, and the $\beta 2$ subunit contains one PKC site in this region (Goldman et al., 1987; Deneris et al., 1988). One of these sites on the $\alpha 4$ 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 $\alpha 4$ receptor subunit was created in which serine 336 was replaced with alanine. This subunit, denoted $\alpha 4^{\text{S336A}}$, was expressed in oocytes along with a wild-type $\beta 2$ subunit. These mutant receptors formed ion channels that were activated and desensitized by nicotine ($10 \mu\text{M}$) in a manner similar to wild-type $\alpha 4\beta 2$ nAChRs (Fig. 4). Dose-response relationships in the presence of Ca^{2+} yielded an EC_{50} value of $13 \mu\text{M}$, also similar to wild-type $\alpha 4\beta 2$ receptors (Fenster et al., 1997). The onset of desensitization (nicotine $10 \mu\text{M}$; 2 min) could be described by a single (1/6 cells) or a biexponential decay (5/6 cells), with fast [$\tau_f = 6.9 \pm 1.6$ s (24%)] and slow ($\tau_s = 135 \pm 20$ s) time constants; these data are not significantly different from wild-type receptors [$\tau_f = 5.1 \pm 0.4$ s (24%); $\tau_s = 109 \pm 22$ s]. The magnitude of desensitization in $\alpha 4^{\text{S336A}}\beta 2$ nAChRs, estimated at the end of a 2-min nicotine applica-

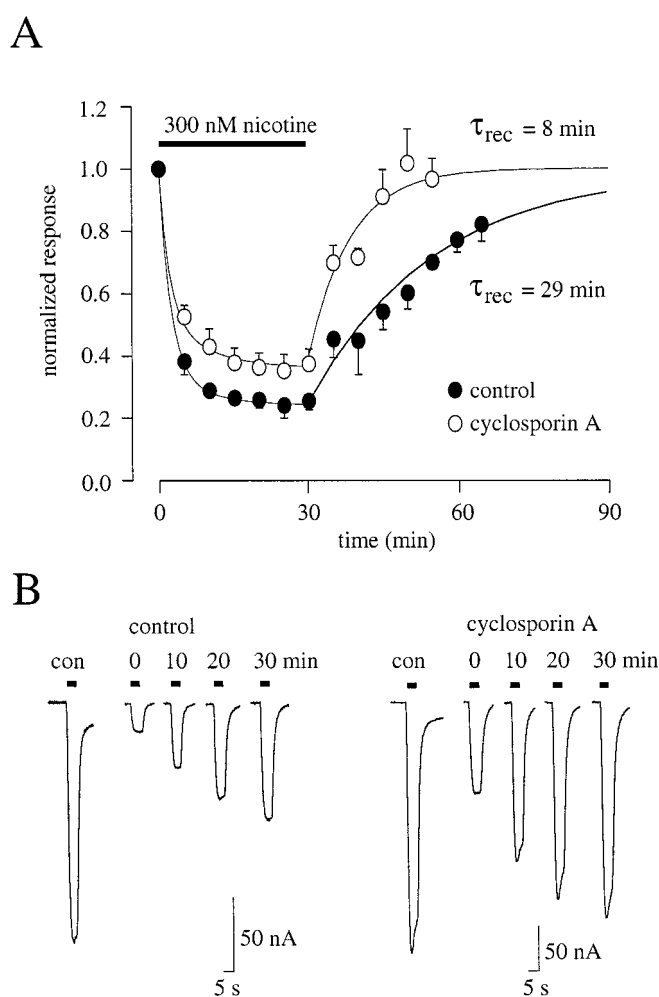


Fig. 3. Phosphatase inhibition increases the rate of recovery from desensitization. The time courses of desensitization and recovery during a 30-min incubation with 300 nM nicotine in $\alpha 4\beta 2$ -expressing oocytes injected with cyclosporin A. These oocytes were compared with control oocytes (uninjected/vehicle-injected) 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 before nicotine application. The solid lines show double exponential fits to the onset of desensitization with fast (τ_f) and slow (τ_s) 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.

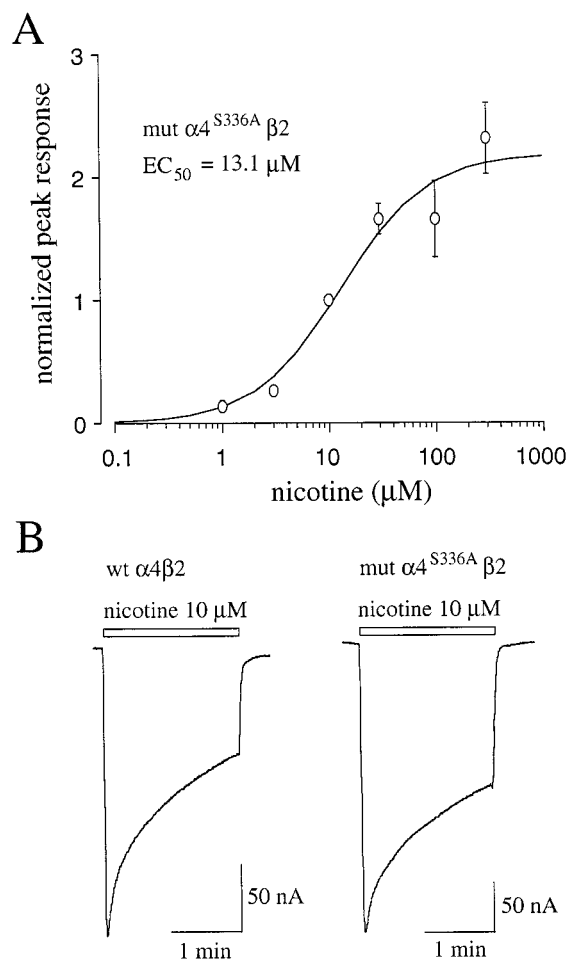


Fig. 4. Properties of mutant $\alpha 4^{\text{S336A}}\beta 2$ receptors. A, concentration-response relationship for activation of mutant $\alpha 4^{\text{S336A}}\beta 2$ receptors. The solid curve is a logistic fit to the data, with a Hill slope of 1.0. B, comparison of currents induced by 2-min applications of nicotine in wild-type $\alpha 4\beta 2$ - (left) and $\alpha 4^{\text{S336A}}\beta 2$ - (right) expressing oocytes.

tion, was less than in wild-type $\alpha 4\beta 2$ receptors: 0.46 ± 0.02 ($n = 6$) compared with 0.59 ± 0.02 ($n = 7$; $P < .05$).

Recovery from desensitization for wild-type $\alpha 4\beta 2$ receptors was compared with recovery for $\alpha 4^{S336A}\beta 2$ receptors. Assuming that the mutation at this site on the $\alpha 4$ subunit interferes with the ability of kinases and phosphatases to modulate recovery from desensitization, then the mutant receptor should show slowed recovery from desensitization. Fig. 5 shows that although the mutation did not markedly affect the onset of desensitization, as expected from the brief pulses (Fig. 4B), 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 [$\tau_{rec}(\text{fast}) = 7.2$ 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. 2B). These data imply that other factors may be important for regulation of $\alpha 4\beta 2$ 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 $\alpha 4^{S336A}\beta 2$ receptors should not be enhanced by

PMA treatment. In the presence of both PMA and cyclosporin A, the overall extent of recovery from desensitization of mutant nAChRs was enhanced, as judged from the appearance of a slow phase of desensitization [$\tau_{rec}(\text{slow}) = 63$ min; data not shown] that was absent in untreated mutant receptors (Fig. 5). The fast phase of recovery from desensitization was unaffected by this treatment [$\tau_{rec}(\text{fast}) = 9.0$ min]. Because cyclosporin A and PMA did not enhance recovery from desensitization of mutant nAChRs [$\tau_{rec}(\text{slow}) = 63$ min] to the same extent as wild-type receptors ($\tau_{rec} = 30$ 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 $\alpha 4\beta 2$ Receptor Desensitization. The most convenient method for understanding how phosphorylation could regulate $\alpha 4\beta 2$ 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):

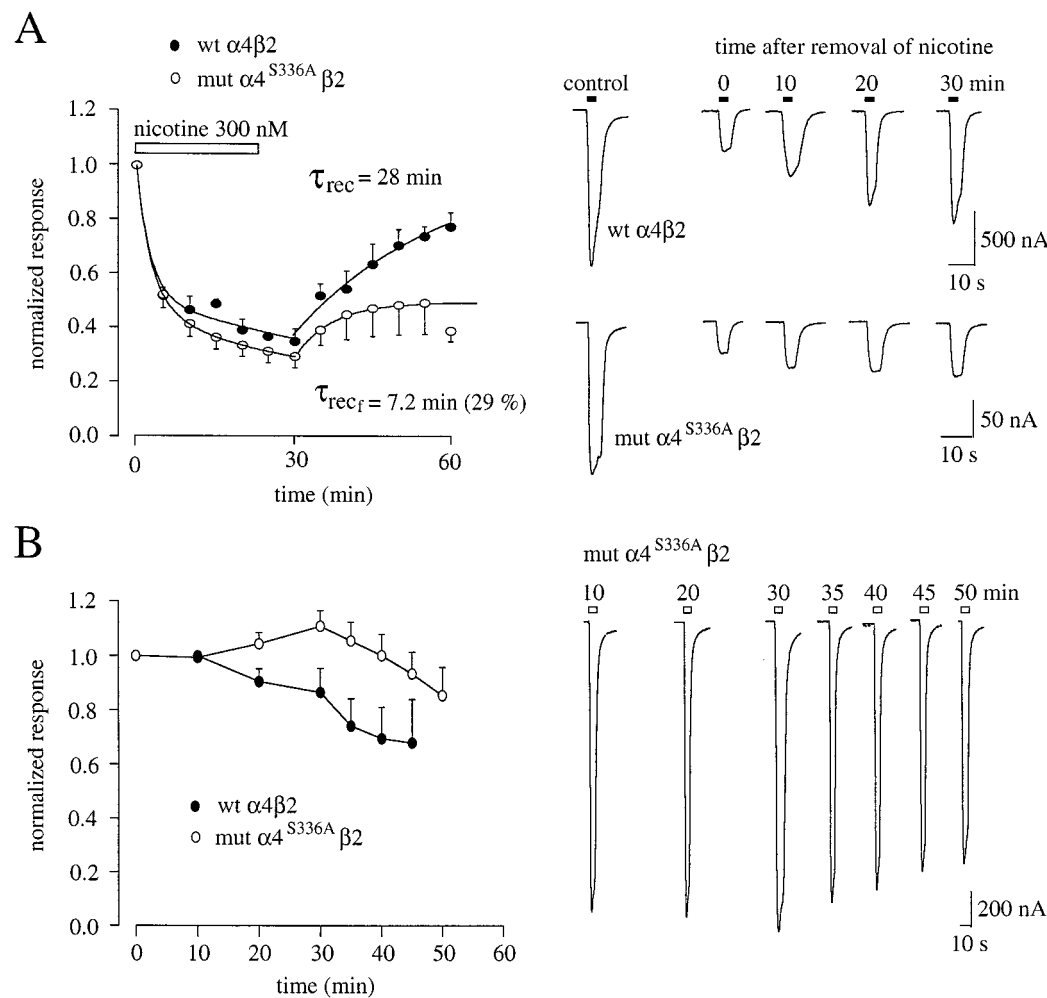
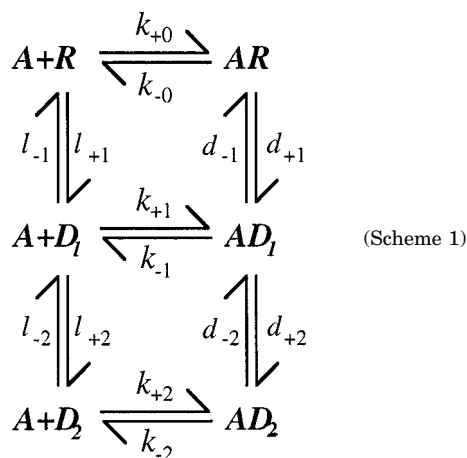


Fig. 5. Recovery from desensitization is reduced in mutant $\alpha 4^{S336A}\beta 2$ receptors. **A**, the time courses of desensitization and recovery were assessed during a 30-min incubation with 300 nM nicotine in wild-type $\alpha 4\beta 2$ (filled symbols) or mutant $\alpha 4^{S336A}\beta 2$ (open symbols) 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 $\alpha 4\beta 2$ and mutant $\alpha 4^{S336A}\beta 2$ receptors. Recovery from desensitization of wild-type $\alpha 4\beta 2$ receptors has been fit to a single exponential with its time constant shown. Recovery from desensitization of mutant $\alpha 4^{S336A}\beta 2$ receptors was best fit with the sum of two exponentials. **B**, Initial stability of peak amplitudes of test pulses. Brief pulses of nicotine (10 μ M) were applied at 5- and 10-min intervals to oocytes expressing either wild-type $\alpha 4\beta 2$ (filled symbols; $n = 10$) or mutant $\alpha 4^{S336A}\beta 2$ (open symbols; $n = 11$). Time-course plots of the test pulse amplitudes (left) and example responses (right) are shown.



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, after removal of agonist, both of these 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 $D_1 \rightarrow R$ and $D_2 \rightarrow D_1$, 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 $\alpha 4\beta 2$ nAChRs expressed in oocytes is $10 \mu\text{M}$ (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 $\approx 60\%$ of receptors are “instantaneously” ($< 2 \text{ min}$) desensitized (Table 1), the fast component of desensitization must proceed via the transition $R \rightarrow D_1$. 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 also Feltz and Trautmann, 1982):

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

At 300 nM nicotine, $R/R_{\max} \approx 0.4$ (Fig. 1). If $K_1 = 100 \text{ nM}$ (a high enough affinity for interaction with nanomolar concentrations of nicotine), then $L_1 \approx 1$. The individual rates l_{+1} and l_{-1} can be calculated based on the observation that the equilibration of R and D_1 is fast [i.e., $\tau_f \approx 1 \text{ min}$ (Table 1)]:

$$\tau_f = \frac{1}{l_{+1} + l_{-1}} \quad (2)$$

In this case $l_{+1} = l_{-1} = 0.5 \text{ min}^{-1}$. 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. 7F). The time constant τ_f for

this process ($\approx 5 \text{ s}$; see Fig. 4) is related to the rate constants by:

$$\tau_f = \frac{1}{f \cdot d_{+1} + d_{-1}} \quad (3)$$

At $10 \mu\text{M}$ 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 eq. 3 gives $d_{+1} = 24 \text{ min}^{-1}$ and $d_{-1} = 0.24 \text{ min}^{-1}$. After the initial fast phase of desensitization, most receptors will be in AD_1 and the slow component of desensitization will reflect the transition $AD_1 \leftrightarrow AD_2$. 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, $\tau_s = 15 \text{ min}$, $d_{+2} = 0.064 \text{ min}^{-1}$, and $d_{-2} = 0.0032 \text{ min}^{-1}$. Provided that equilibrium [^3H]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 0.2 (see eq. 4). The individual allosteric rates that determine the slow rate of recovery from desensitization can be calculated from eq. 2 assuming a slow recovery time constant, $\tau_{\text{rec}} = 30 \text{ min}$. Assuming again that [^3H]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 $\approx 0.5 \text{ min}^{-1}$ 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 s 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. 6A) was often observed in the oocyte experiments (see Fig. 5B). A 30-min exposure of the model to 300 nM nicotine produced a biphasic onset and recovery from desensitization with appropriate time constants (Fig. 6B). After 20-min applications of various concentrations of nicotine (Fig. 6C, arrow), a pseudo-steady-state desensitization dose-response curve was constructed (Fig. 6D). 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 $D_2 \leftrightarrow D_1$. However, because the rate out of desensitization l_{-2} is 5-fold faster than l_{+2} , it will dominate the overall rate of recovery (see eq. 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 3-fold reduction in l_{-2} produced a slowing of the slow recovery phase with little or no effect on any other process (Fig. 7A; Table 1). Thus, we would argue that the effects of the $\alpha 4$ mutation and the actions of Ba^{2+} can be largely explained by slowing the transition $D_2 \rightarrow D_1$. 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 $AD_2 \rightarrow AD_1$ was 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 3-fold 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 effect of the mutation/dephosphorylation is to slightly reduce the maximal response that can be generated (see Fig. 7F).

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 (Table 2). 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 $D_2 \rightarrow D_1$ (Fig. 7B). However, this manipulation alone does not predict the PMA- and cyclosporin A-induced changes in the magnitude of desensitization (Fig. 2A). Slowing the rate constant d_{+2} that controls the onset of desensitization $AD_1 \rightarrow AD_2$ (and l_{+2} ; $D_1 \rightarrow D_2$) 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. 7C). 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 Fig. 7, D and E, respectively. In the case of PMA, the rate of recovery ($D_2 \rightarrow D_1$) is enhanced by a 2-fold increase in l_{-2} and the magnitude of desensitization ($AD_1 \rightarrow AD_2$) is reduced by a 2-fold 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. 2B and Fig. 5A).

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 $\alpha 4\beta 2$ nAChRs expressed in *X laevis* 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 $\alpha 4$ subunit, enhance the overall rate of recovery from desensitization.

Desensitization of $\alpha 4\beta 2$ 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 desensitization properties and one with slow desensitization properties (for example, see Maconochie and Knight, 1992). 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). After 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

TABLE 1

Model rate constants

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

Rate Constant	Control	$\alpha 4^{\text{S336A}}\beta 2$ (Fig. 7A)	PMA (Fig. 7D)	Calphostin C (Fig. 7E)
<i>min</i> ⁻¹				
k_{+0}	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

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 $\alpha 4\beta 2$ receptor desensitization occurs rapidly (< 2 min) and contributes $> 60\%$ of desensitization onset at 300 nM nicotine, $\approx 50\%$ 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 $R \rightarrow D_1$ 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 $\approx 80\%$ of $\alpha 4\beta 2$ 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 desensiti-

zation 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 Boyd's 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, $\alpha 4\beta 2$ 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 $D_2 \rightarrow D_1$. The slower rate of recovery from desensitization in Ba^{2+} -containing media implies that Ca^{2+} may be important for normal $\alpha 4\beta 2$ 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 recep-

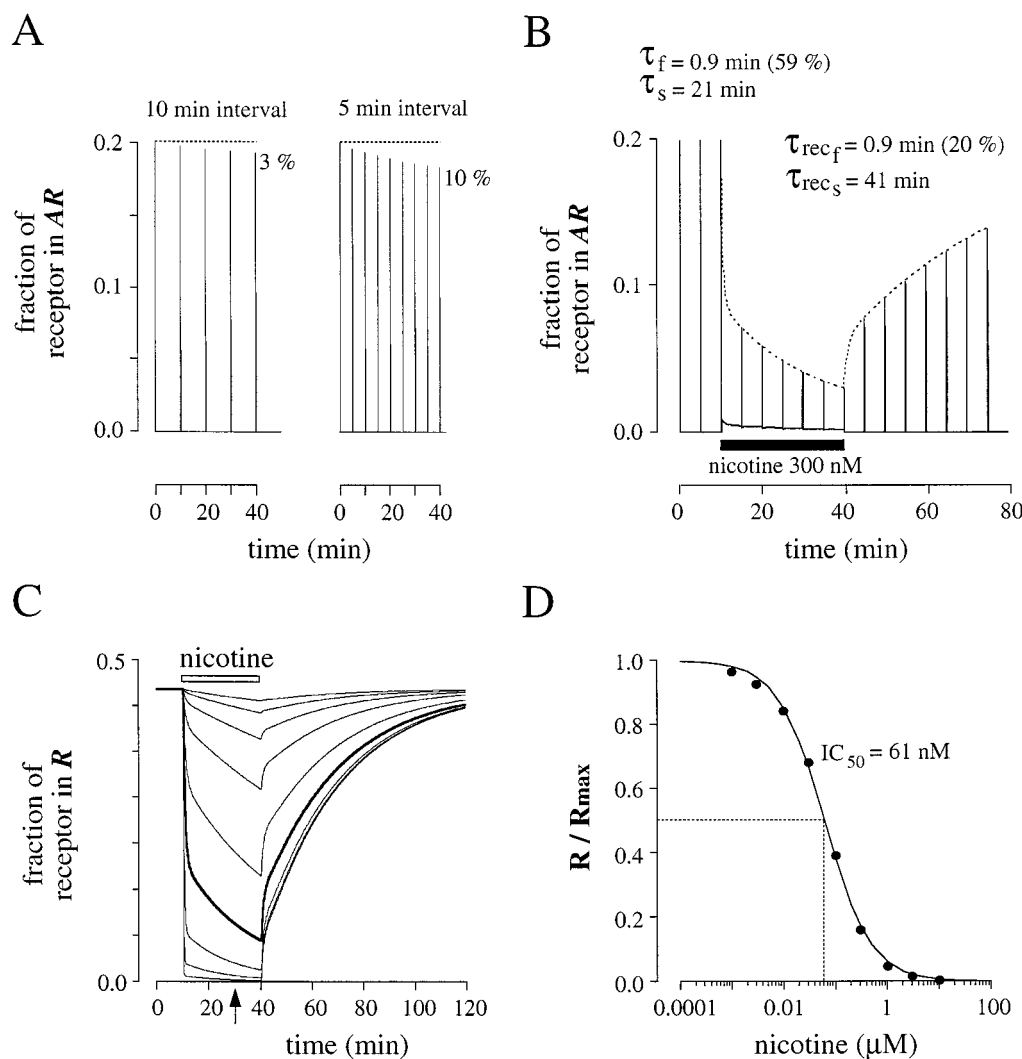


Fig. 6. Simulation of $\alpha 4\beta 2$ receptor desensitization. **A**, initial stability of test pulse amplitude. Simulated responses to 10-s applications of nicotine (10 μM) at 10-min (left) or 5-min (right) intervals. The percentage loss of response is shown in each case after 20 min of stimulation. **B**, simulated desensitization experiment using 2-s pulses of nicotine (10 μM) applied at 5-min intervals before, during, and after exposure to 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 responses 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 nicotine concentration. The solid line is a logistic fit to the simulated data.

tor (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 $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ recep-

tors are almost identical with the behavior of the mutant $\alpha 4^{\text{S336A}}\beta 2$ 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.

$\alpha 4$ Subunits are Potential Substrates for PKC. Direct phosphorylation of $\alpha 4$ 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 $\alpha 4^{\text{S336A}}\beta 2$ nAChR exhibited a decrease in the slow

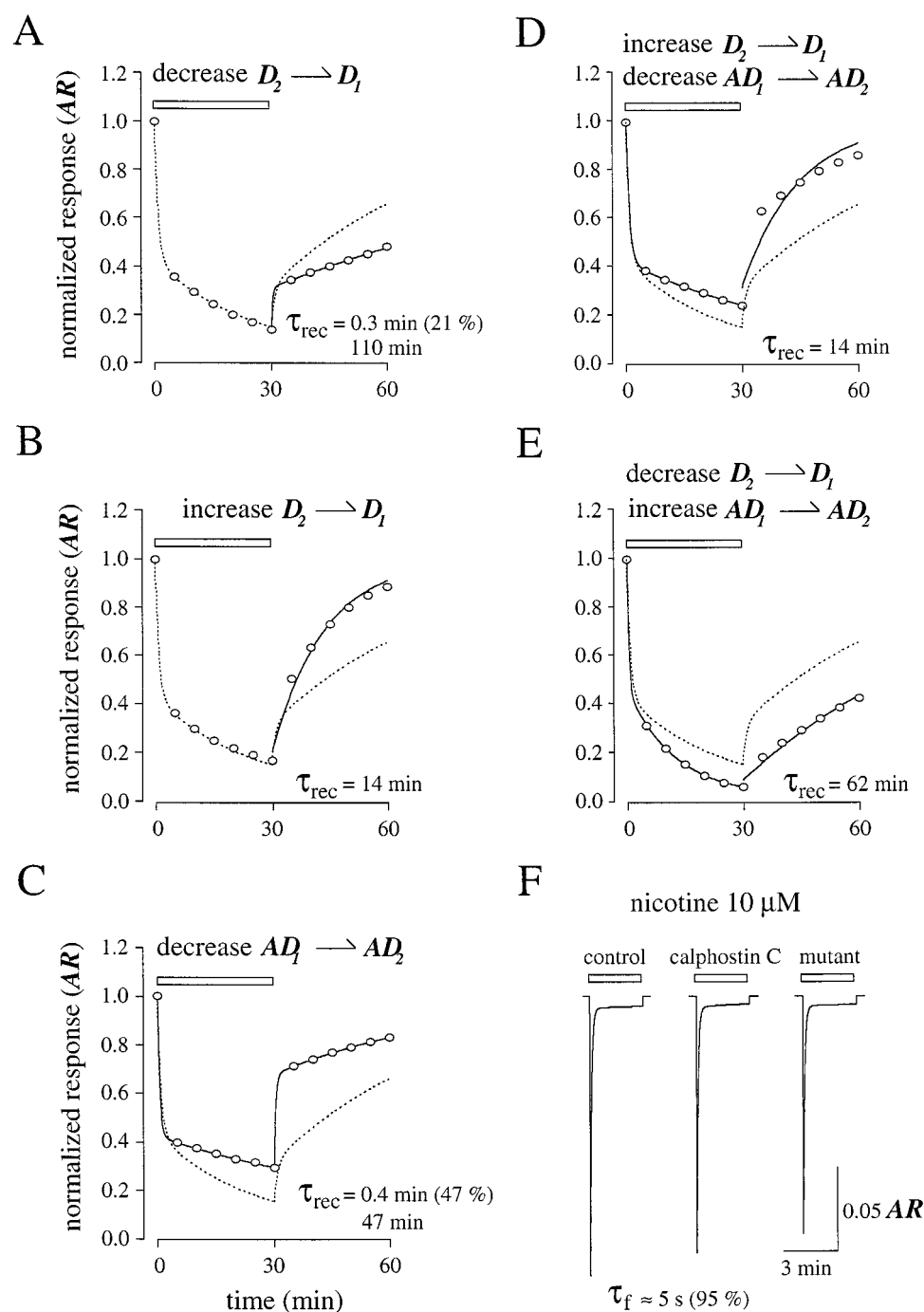


Fig. 7. The effects of rate-constant changes on the time course of desensitization. A, simulated test pulse (nicotine, 2 s; $10 \mu\text{M}$) amplitudes during and after a 30-min simulated exposure to 300 nM nicotine. The rate of recovery from desensitization is slowed by a 3-fold reduction in l_{-2} (d_{-2} was reduced by a similar amount to maintain microscopic reversibility). A 3-fold increase in l_{-2} (and d_{-2}) increased the rate of recovery (B), whereas a 3-fold 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 2-fold increase in l_{-2} balanced by a 2-fold decrease in d_{+2} enhanced the rate of recovery and decreased the magnitude of desensitization. E, a 2-fold decrease in l_{-2} balanced by a 2-fold 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; $10 \mu\text{M}$). 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 three simulations.

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., 1994) 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. 7F). 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 $\alpha 4$ subunits. Indeed the $\alpha 4$ subunit alone contains five 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 $\alpha 4$ 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 $\alpha 4\beta 2$ receptor desensitization, together with the experimental data, these results imply that there is more than one site of action of PKC on $\alpha 4\beta 2$ 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 that phosphorylation can regulate native $\alpha 4\beta 2$ 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 up-regulates the number of high-affinity ($\alpha 4\beta 2$) 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 $\alpha 4\beta 2$ nAChRs have an intrinsically slow rate of recovery from desensitization after a 30- to 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 $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ nAChRs to a functionally inactive conformation (Eilers et al., 1997). Chronic PMA treatment, which down-regulates PKC activity (Favaron et al., 1990), promotes an increase in the number of $\alpha 4\beta 2$ receptors (Gopalakrishnan et al., 1997), consistent with the suggestion that the dephosphorylated state of the receptor could either directly or indirectly serve as a signal for preventing receptor turnover (Peng et al., 1994).

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