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Desensitization of nicotine acetylcholine receptors: Modulation by kinase activation and phosphatase inhibition

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Abstract

The desensitization of α-bungarotoxin-insensitive native neuronal nicotinic receptors was studied in rat cortical cell cultures using the patch clamp technique. Thirty-minute perfusions of nicotine reduced currents evoked by short test pulses of 300 μM acetylcholine over a range of 3 to 300 nM, with an IC_{50} of 51 nM. The time course of desensitization onset was fit by a biexponential function consisting of a fast time constant of about 1 min and a slower component of 6–10 min. The desensitization recovery process was also biexponential and was dominated by a slow time constant of 12–20 min, as well as a minor component of about 1 min. The intracellular dialysis of either the protein kinase C activator phorbol-12-myristate-13 acetate or the phosphatase inhibitor cyclosporin A accelerated the desensitization recovery rate by 2-fold. The data imply that endogenous cortical nicotinic receptor channels may enter one of two desensitization states. The first state (D_1) is characterized by rapid entry and recovery, whereas transitions into and out of the second state (D_2) occur at slower rates. The D_2 receptor state may arise by a sequential transition from the D_1 conformation. Protein kinase C activation or phosphatase 2B inhibition may favor the D_1 receptor state over that of D_2 to promote faster overall rates of desensitization recovery.

Keywords: Nicotine; Desensitization; Acetylcholine; Nicotinic receptor; Smoking; Phosphorylation

1. Introduction

Since the "discovery" in pre-Columbian America nearly 500 years ago, tobacco-related products have spread to nearly every corner of the globe and are regularly used by 1.1 billion smokers. However, it is only within the last 30 years that science has begun to understand the molecular mechanisms of how nicotine, the primary constituent of tobacco, exerts its effects on the brain. It is now clear that nicotine must be interacting with one or more of the multiple subtypes of neuronal nicotinic acetylcholine receptors that are formed from various homo- and heteromeric combinations of α and β nicotinic subunits (McGehee and Role, 1995). Perhaps the most pertinent neuronal nicotinic receptors related to nicotine use are those composed of the $\alpha_4\beta_2$ subunit combination. The $\alpha_4\beta_2$ nicotinic receptor is the predominant high-affinity nicotine binding receptor in the mammalian brain (Flores et al., 1992). Furthermore, $\alpha_4\beta_2$ nicotinic receptor densities increase following chronic exposure to low concentrations of nicotine—both in vivo (Flores et al., 1992) and in vitro (Peng et al., 1994; Whitaker et al., 1998). Such characteristics mark them as prime mediators of the effects of tobacco on the central nervous system.

Pharmacokinetic studies of smokers indicate that venous nicotine concentrations gradually increase over the course of a day as a series of "peaks and troughs" until a steady-state plateau is reached (Russell, 1987). However, even in the heaviest smokers these plateaus rarely reach 300 nM. Even the measured "per puff" arterial nicotine bolus reaching the brain from the lungs is usually less than 1 μM. (Henningfield et al., 1993). In cultured cortical cells, currents evoked by the acute application of 300 nM nicotine are less than 5% of the maximally attainable acetylcholine-induced current

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(Marszalec et al., 1999). However, a 10 min perfusion of 300 nM nicotine desensitizes acetylcholine test responses by more than 60%. Such in vivo and in vitro observations have prompted speculation that some behavioral effects related to tobacco use arise from $\alpha_4\beta_2$ nicotinic receptor desensitization (Balfour, 1994).

In this study, we have recorded and analyzed the nicotine-induced desensitization of α-bungarotoxin-insensitive acetylcholine-evoked currents in rat cortical neurons. In addition to studying the magnitude and temporal aspects of nicotine-induced desensitization, additional experiments were conducted to determine whether agents modifying the activities of intracellular protein kinase C or phosphatase 2B might alter the character of this desensitization. This is in keeping with literature reports that agents that modify protein phosphorylation also modify nicotinic receptor desensitization (Swope et al., 1999). Studies in cell lines and transfected *Xenopus* oocytes show that $\alpha_4\beta_2$ receptor desensitization is affected by either protein kinase C activation or phosphatase inhibition (Eilers et al., 1997; Khiroug et al., 1998; Fenster et al., 1999). We wanted to determine whether this was also true for $\alpha_4\beta_2$-like nicotinic receptors in native cortical neurons. The use of cultured neurons allows experiments of nicotinic receptors in their native membrane environment, while avoiding some of the problems arising from the transfection of these receptors in foreign hosts (Lewis et al., 1997; Sivilotti et al., 1997; Buisson et al., 2000).

2. Materials and methods

2.1. Primary cultures of cortical neurons

Cortical neurons were prepared from 17-day embryonic Sprague–Dawley rat pups removed under methoxyflurane anesthesia as approved by the Northwestern University Animal Care and Use Committee. The brains were dissected in calcium- and magnesium-free phosphate buffered saline solution (PBS). Small wedges of frontal cortex were placed in PBS containing 0.25% (w/v) trypsin (type XI, Sigma-Aldrich, St. Louis, MO) and incubated for 20 min at 35 °C. Afterwards, the tissue was triturated by repeated passage through a flame-narrowed bore of a Pasteur pipette. The dispersed cells were suspended in a serum-free Neurobasal medium with B-27 supplement (Invitrogen, Carsbad, CA) and 2 mM glutamine added. The density was adjusted to 100,000 cells/ml from which 3 ml was aliquoted into 35-mm culture wells. Each well contained five 12-mm poly-L-lysine-coated glass coverslips that had been seeded with glia 2 to 4 weeks earlier (Marszalec and Narahashi, 1993). This neuronal/glia co-culture was maintained in a humidified atmosphere of 93% air/7% CO_2 for up to 8 weeks. Cytosine-D-abrabinofuranoside (8 μM) was added to the culture media after the first week to inhibit glial overgrowth. Half of this media was replaced once a week. Experiments were conducted on cells cultured for 3 to 5 weeks.

2.2. Electrophysiological recordings

Whole-cell currents were recorded by standard patch clamp recording techniques with an Axopatch-1C patch clamp amplifier (Molecular Devices, Sunnyvale, CA) at room temperature and a holding potential of −70 mV. Acetylcholine-activated currents were digitized at 1–10 kHz and acquired to the computer hard disk by a Digidata 1200 ADC/DAC interface under the control of a PClamp software program (Molecular Devices). The series resistance of the recording pipette/cell pathway was monitored regularly by a short pulse to −10 mV. If the series resistance deviated from the control value by more than 20%, the data were discarded.

2.3. Solutions and drugs

The external solution contained (in mM): 150 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 5.5 HEPES, 4.5 Na-HEPES, pH 7.4. The solution also contained 100 nM tetrodotoxin (International Wex Technologies, Vancouver, B.C.) to block spontaneous action potentials. Other chemicals (all from Sigma-Aldrich, St. Louis, MO unless otherwise noted) included 10 μM picrotoxin and 1 μM 6-cyano-7-nitroquinoxaline-2,3-dione to block γ-aminobutyric acid (GABA) and glutamate currents, respectively. Atropine sulphate (20 nM) was added to block muscarinic receptors. Finally, 25 nM α-bungarotoxin was present in all external solutions to prevent the activation of α7-like nicotinic receptor-mediated currents. In some experiments 100 μM phorbol-12-myristate-13 acetate or 500 nM cyclosporin A was added to the pipette prior to seal formation and membrane rupture.

The patch clamp pipettes were pulled from Kimax borosilicate glass tubing (Kimble Glass Inc., Vineland, NJ) and filled with an internal solution containing (in mM): 140 Cs gluconate, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 HEPES acid, 2 Mg^{2+} ATP, and 0.2 Na^+ GTP, pH 7.3 adjusted with CsOH.

Acetylcholine was directly applied to the cell by means of a computer-driven solenoid U-tube perfusion device with a solution exchange time of 10 to 20 ms (Marszalec and Narahashi, 1993). Nicotine was perfused through the bath and simultaneously applied with ACh via the U-tube.

2.4. Data analysis

All current amplitudes were measured by the ClampFit module of the PClamp6 software program. The rates of desensitization onset and recovery were estimated by a non-linear analysis curve-fitting program of the SigmaPlot software (Jandel Corporation, San Rafael, CA). The significance of differences produced by various treatments was assessed by Student's *t*-test for unequal sample sizes.

3. Results

3.1. Acetylcholine-induced currents in cultured cortical neurons

Data previously reported by this laboratory (Aistrup et al., 1999) have shown that two types of acetylcholine-induced currents can be recorded from cortical neurons. One type of acetylcholine-evoked response rapidly desensitized ($\tau \approx 20$ ms) at high concentrations of agonist and was inhibited by nanomolar levels of α-bungarotoxin. The second type of current desensitized more slowly ($\tau \approx 5$ s) at high levels of agonist, was unaffected by α-bungarotoxin, but was antagonized by dihydro-β-erthroidine. The acetylcholine EC_{50}s for α-bungarotoxin-sensitive and -insensitive currents were 330 μM and 2.7 μM, respectively. Nicotine activated the α-bungarotoxin-insensitive currents with an EC_{50} of 3.4 μM (Marszalec et al., 1999). Overall, the pharmacological profile observed with α-bungarotoxin, nicotine, dihydro-β-erthroidine and other agents (Aistrup et al., 1999) implicate the receptors underlying the α-bungarotoxin-insensitive response as those of the $\alpha_4\beta_2$ subtype (Luetje and Patrick, 1991; Chavez-Noriega et al., 1996).

Cortical neurons exhibiting α-bungarotoxin-insensitive currents were found in a subgroup of non-pyramidal-shaped cells. These $\alpha_4\beta_2$-like responses were most often recorded by focusing on smaller spindle-shaped neurons. These $\alpha_4\beta_2$-like currents were not prominent until 3 to 4 weeks after cell culture.

Nicotine desensitization was monitored on currents evoked by 250 ms pulses of 300 μM acetylcholine applied every 1 to 5 min. Fig. 1 shows that a 10 s application of 300 μM acetylcholine desensitized putative $\alpha_4\beta_2$ responses, reducing the peak current amplitude by an average of 61±10% (mean±S.E.M., $n=25$). This desensitization event

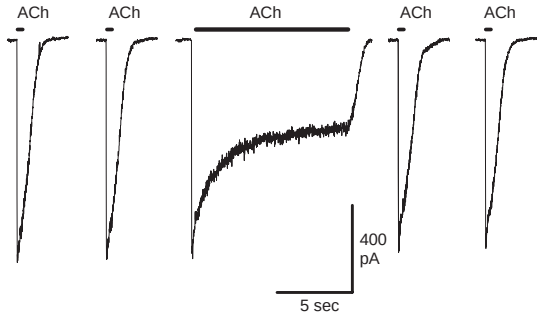

Fig. 1. Acetylcholine (ACh) activates and desensitizes cortical cell nicotinic receptors. Whole-cell currents were activated by U-tube applications of 300 μM acetylcholine in the presence of 25 nM α-bungarotoxin at a holding potential of −70 mV. Each acetylcholine pulse was separated by intervals of 1 min. The shorter currents result from 250 ms acetylcholine applications, whereas in the middle trace the agonist was applied for 10 s. The 10 s acetylcholine application sequentially activated and desensitized the nicotinic receptors to decrease the peak current response by about 60%. Afterwards, the peak response recovered to the control amplitude within 1 min.

was a double exponential process having both a fast and a slow τ value averaging 638±167 ms and 4.6±3.7 s, respectively ($n=20$). However, Fig. 1 indicates that this acute desensitization of the peak current fully recovered (97.2±1.0% $n=6$) within a period of 1 min. Therefore, it is unlikely that the acetylcholine test pulse paradigm itself desensitizes the nicotinic receptors. In some neurons, the control responses have remained steady (rundown<5%) for up to 2 h.

3.2. Nicotine-induced receptor desensitization and recovery

The magnitude and rate of nicotine-induced nicotinic receptor desensitization were measured by a protocol similar to that shown in Fig. 2. First, several 300 μM acetylcholine test pulses were applied over a 15 min control period before the perfusion of nicotine to measure the control amplitude and ensure the stability of the undesensitized response. Next, a perfusion of nicotine was introduced into the recording chamber (solution exchange time of about 15 s) and continued for 30 min. During this perfusion, acetylcholine pulses were applied in the presence of nicotine. Initially, these co-applications were repeated every 5 min in keeping with the oocyte study reported by Fenster et al. (1999). However, it was found that for cortical neurons test pulses had to be applied every min for each of the first 3 min during both introduction and washout of nicotine to adequately estimate the faster components of desensitization onset and recovery. After nicotine was removed from the bath, the desensitization recovery was monitored for up to 1 h or to 95% recovery, whichever came first.

Several sets of experiments were conducted using this protocol for nicotine concentrations ranging from 3 to 300 nM. Currents recorded at different experimental time intervals were normalized as a fraction of the peak control response. These were averaged for several cells and plotted for different nicotine concentrations as shown in Fig. 3. Only the highest concentration of bath perfused nicotine (300 nM) evoked an inward current of its own—averaging 7.3±0.6% of acetylcholine test response ($n=7$) (see also Marszalec et al., 1999). This small nicotine-induced current (which also desensitized over time) was added to the control response amplitude in calculating the amount of receptor desensitization in Fig. 3.

The averaged time points here (mean±S.E.M.) are joined by best-fit curves that estimate the rates of onset and recovery at the given concentrations of nicotine. The exponential τ values for these curves are given in Table 1. The desensitization onset rate at all nicotine concentrations was composed of two exponentials. The range of the fast onset component ($\tau_{on\text{-}fast}$) over the nicotine concentrations tested ranged from 0.5 to 0.9 min, whereas the range of the slow components ($\tau_{on\text{-}slow}$) was from 6.1 to 11.3 min. Both Fig. 3 and Table 1 indicate that the proportion of the faster component in the overall rate of desensitization onset was largest at 100 and 300 nM nicotine, whereas the proportion

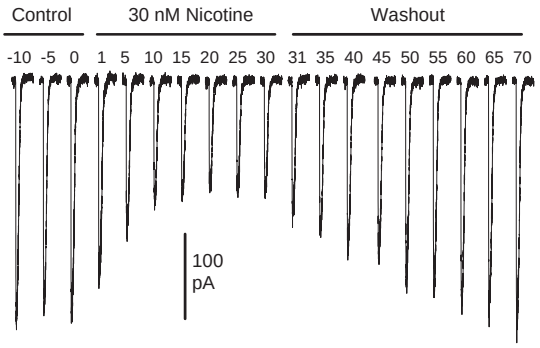

Fig. 2. An extended perfusion of nicotine at low concentrations desensitizes cortical α-bungarotoxin-insensitive acetylcholine currents with a slow onset and recovery. Sequential current records were evoked in the same cell by 250 ms applications of 300 μM acetylcholine. The first three records represent control responses given at the indicated times (in min) prior to the start of a 30 min bath perfusion of 30 nM nicotine. Over the 30 min time course shown here the perfusion of nicotine diminished the amplitude of the acetylcholine response, rapidly at first and then more slowly. At the end of 30 min, nicotine was removed from the bath and the response began to gradually recover, returning to the original peak current level at 70 min.

of the slower component was nearly constant at all concentrations.

At the end of all 30 min perfusions there remained a residual undesensitized current that decreased as the nicotine concentration was increased. These residual currents were normalized as a fraction of the pre-nicotine control response and are listed in Table 1. A plot of these values (inset Fig. 3) estimated the desensitization IC_{50} after 30 min nicotine exposure to be 51 nM.

The time course for desensitization recovery for most nicotine concentrations was best fit by a double exponential function (although for some concentrations a single exponential may also have sufficed). The fast recovery components ($\tau_{off\text{-}fast}$) ranged from 0.1 to 1.2 min, whereas the prominent slow components ($\tau_{off\text{-}slow}$) ranged from 17 to 27 min. The proportion of the fast recovering component was very small. Instead the overall rate of receptor recovery from nicotine-induced desensitization was governed by the slow exponential component.

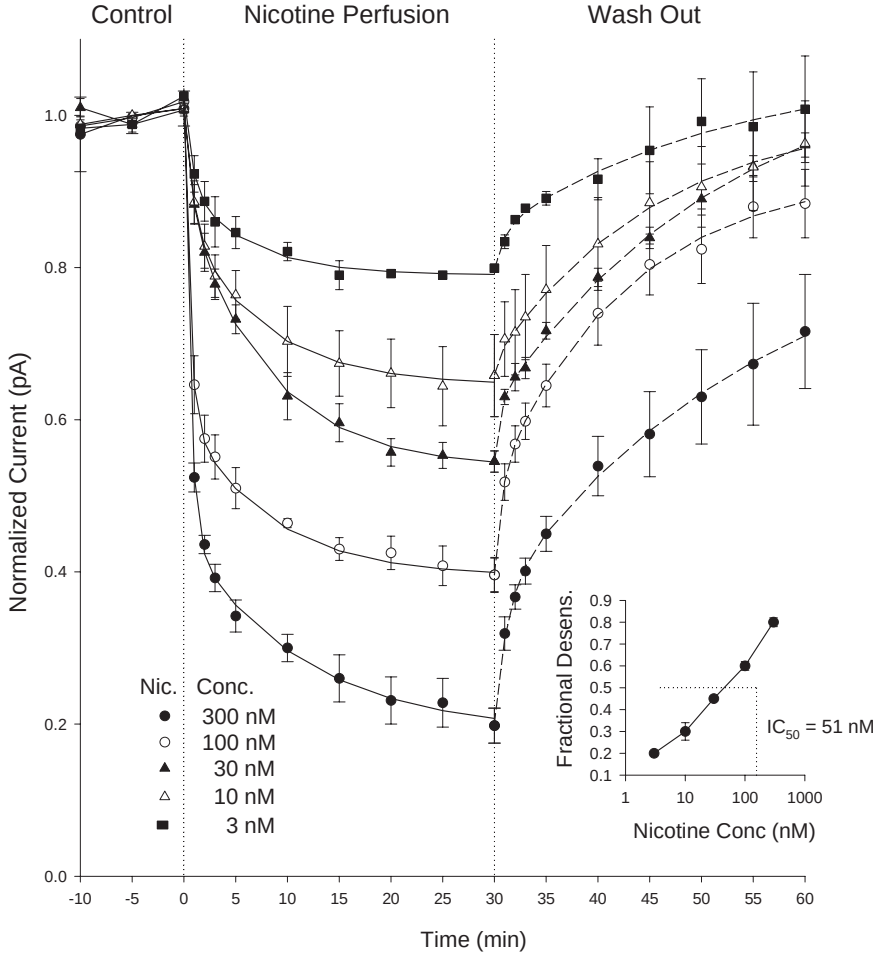

Fig. 3. Nicotine desensitizes cortical cell α-bungarotoxin-insensitive nicotinic receptors in a dose-dependent manner. The average normalized peak current amplitudes evoked by 250 ms pulses of 300 μM acetylcholine pulses are plotted before, during, and after a 30 min perfusion of nicotine at the concentrations indicated. The current records for each individual experiment were normalized to the peak current amplitude recorded before the perfusion of nicotine. Each point represents the mean ± S.E.M. for a given concentration of nicotine. The lines connecting the points represent best fits generated by a curve-fitting program. Both the onset and recoveries were fit by biexponential functions. (Inset) Dose–response curve for the fractional desensitization of the acetylcholine-induced test response at 30 min. The IC_{50} for nicotine-induced desensitization was 51 nM.

Table 1
Analysis of onset and recovery rates for nicotine-induced receptor desensitization and their modification by kinase activation or phosphatase inhibition

Nicotine	*n*	Residual current	Onset		Recovery	
			$\tau_{on\text{-}fast}$ min (fraction)	$\tau_{on\text{-}slow}$ min (fraction)	$\tau_{off\text{-}fast}$ min (fraction)	$\tau_{off\text{-}slow}$ min (fraction)
300 nM	4	0.20±0.02	0.6 (0.566)	11.3 (0.260)	1.2 (0.166)	26.8 (0.511)
100 nM	5	0.40±0.02	0.5 (0.410)	8.8 (0.214)	0.7 (0.172)	20.0 (0.437)
30 nM	6	0.55±0.02	0.8 (0.116)	7.9 (0.335)	0.7 (0.071)	22.6 (0.469)
10 nM	6	0.70±0.04	0.9 (0.151)	7.7 (0.211)	0.7 (0.220)	16.8 (0.331)
3 nM	2	0.80	0.8 (0.116)	6.1 (0.410)	1.0 (0.050)	22.4 (0.217)
300 nM	9	0.20±0.02				
300 nM+100 μM PMA	6	0.46±0.07				
300 nM	4	0.20±0.02	0.6 (0.566)	11.3 (0.260)	1.2 (0.166)	26.8 (0.512)
300 nM+500 nM Cyclo-A	5	0.27±0.04	0.6 (0.490)	7.2 (0.250)	0.8 (0.161)	16.0 (0.746)

Equation for onset curve fit: $y=a\cdot e^{(x/-b)}+c\,e^{(x/-d)}+f$.
Equation for recovery curve fit: $y=a(1-e^{(x/-b)})+c(1-e^{(x/-d)})+f$.
y=normalized current amp., x=time in min, a and c=respective fractional proportions for the fast (b) and slow (d) τ values, f=fractional residual current.

3.3. Effects of kinase activity on desensitization

Several reports indicate that the activation of protein kinase C or the inhibition of various phosphatases modify nicotine-induced receptor desensitization (Eilers et al., 1997; Khiroug et al., 1998; Fenster et al., 1999). Therefore, we wanted to determine whether a similar outcome could be observed for $\alpha_4\beta_2$-like nicotinic receptors in cortical neurons. In these experiments, cells were patch clamped with pipette solutions containing 100 μM phorbol 12-myristate 13-acetate (PMA), an agent that activates intracellular protein kinase C. This concentration was kept high to compensate for the rate-limiting step of pipette tip diffusion with respect to the loss of drug via diffusion out the cell (DeCoursey, 1995). In all experiments, the cells were dialyzed with PMA for at least 25 min before the perfusion of nicotine. The results are summarized in Fig. 4 where the effects of PMA were assessed on receptor desensitization evoked by a 30 min application of 300 nM nicotine. In these early experiments, acetylcholine test pulses were given only at 5 min intervals, therefore it was not possible to accurately determine the fast components of the onset and recovery curves.

However, intracellular dialysis of PMA significantly reduced the magnitude of desensitization with an average fractional residual current of 0.458±0.073 (n=6) compared to 0.200±0.021 for the PMA-free control (P<0.05, n=9).

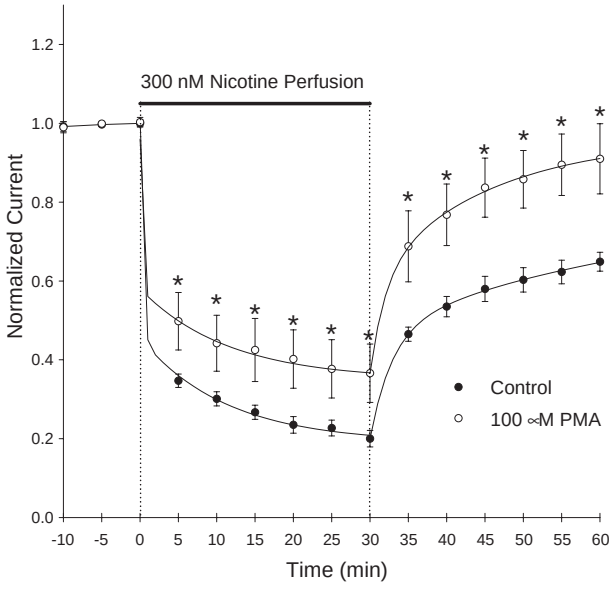

Fig. 4. Phorbol ester decreases the magnitude of nicotinic receptor desensitization and increases recovery rate. The desensitization evoked by a 30 min perfusion of 300 nM nicotine was compared to that observed when cells (n=5) were dialyzed (via the recording pipette) with 100 μM phorbol-12-myristate-13 acetate (PMA), an activator of protein kinase C. This treatment significantly decreased the average magnitude of desensitization over the course of the nicotine perfusion. Following nicotine washout, the recovery of peak current was accelerated by the phorbol ester. Asterisks indicate significant difference of the PMA-treated cells compared to controls (P<0.05).

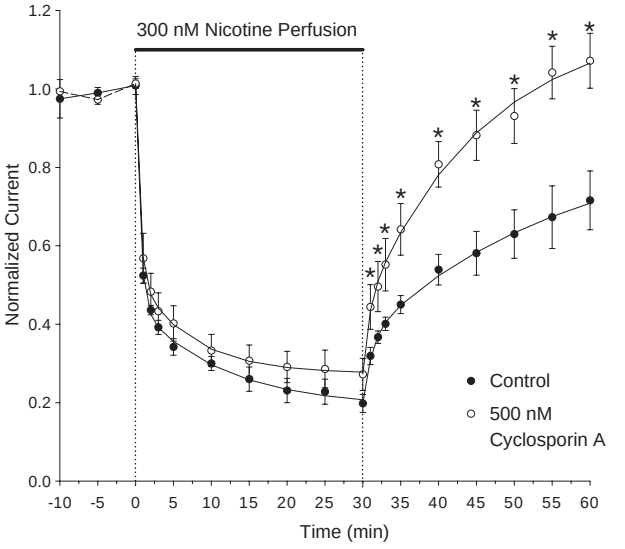

Fig. 5. A phosphatase 2B inhibitor cyclosporin A increases the recovery rate from nicotine-induced receptor desensitization. The graph indicates the effects of the intracellular dialysis of 500 nM cyclosporin A on desensitization evoked by 300 nM nicotine. This treatment significantly increased the rate of receptor resensitization following nicotine washout compared to that of untreated cells (asterisks, P<0.05). Although there was a tendency for cyclosporin A to reduce the maximal desensitization magnitude, this trend was found not significant in this series of experiments.

In addition to this effect, the recovery from desensitization was faster in the presence of PMA as compared to that of the time matched PMA-free controls.

In another set of neurons, the effect of phosphatase inhibitor cyclosporin A was tested by including this compound within the recording pipette solution (Fig. 5). The intracellular dialysis of 500 nM cyclosporin A reduced the magnitude of desensitization as indicated by the fraction of the test current remaining at the end of a 30 min perfusion on 300 nM nicotine (control: 0.20±0.02; $n=5$; cyclosporin A: 0.27±0.04; $n=4$). Despite a smaller effect on the magnitude of desensitization, cyclosporin A greatly increased the recovery rate following nicotine washout. A curve fit of the composite recovery rate shown in Fig. 5 indicates that cyclosporin A decreased the τ value for the slow phase of recovery from 27 min for the untreated controls to 11 min in the treated cells.

4. Discussion

This study extends previous descriptions of nicotinic receptor desensitization (Fenster et al., 1997, 1999) to include those α-bungarotoxin-insensitive $\alpha_4\beta_2$-like receptors endogenous to rat cortical neurons. The desensitization here was evoked by nicotine concentrations similar to those encountered with tobacco use. The original model of motor end-plate activation by Katz and Thesleff (1957) postulated that agonist (A) binding to nicotinic receptor channels (R) either induced or stabilized a conformation (AR) that favored ionic conductance. The continued presence of agonist, particularly at high concentrations, revealed that receptors could also enter a desensitized conformation that restricts ion conductivity (AD_1). This model is depicted in the conformational Scheme 1 given below.

Entry into the desensitized state is significantly slower than that of channel activation. In keeping with this model, the α-bungarotoxin-insensitive whole-cell currents recorded in cortical neurons showed a similarly rapid current onset ($\tau \approx 20$ ms) followed by a slowly increasing phase of desensitization when 300 μM acetylcholine was applied over 10 s.

The original model was modified by Feltz and Trautmann (1982) so as to have two interconverting states of desensitization, with one state (D_1) having a relatively rapid entry and recovery rate, and the other (D_2) undergoing significantly slower transitions. This study suggested that upon shorter applications of agonist, the desensitization was dominated by the fast on/off kinetics of D_1, whereas longer applications favored the transition (or stabilization) to the slowly recovering D_2 conformation (as shown in Scheme 2):

The results obtained in cortical cells also support the existence of two states of nicotinic receptor desensitization.

$$A + R \leftrightarrow AR \leftrightarrow AD_1$$

Scheme 1. One-state desensitization model.

$$\begin{array}{ccc} A + R & \leftrightarrow & AR \\ \updownarrow & & \updownarrow \\ A + D_1 & \leftrightarrow & AD_1 \\ \updownarrow & & \updownarrow \\ A + D_2 & \leftrightarrow & AD_2 \end{array}$$

Scheme 2. Two-state desensitization model.

This may be inferred by comparing the desensitization recovery kinetics observed after 10 s applications of 300 μM acetylcholine to those following 30 min perfusions of 100 nM nicotine. Each application desensitizes the α-bungarotoxin-insensitive nicotinic receptor population by approximately 60%. Whereas a full recovery from the shorter agonist application was attained within 1 min, the recovery from the extended nicotine perfusion required 30 min or more. It is presumed that the fast recovery occurs from the D_1 state, whereas the prolonged agonist exposure permits the transition to the slowly recovering D_2 state.

Boyd (1986) also postulated a two-state desensitization model for the nicotinic receptors in PC12 cells. However, noting the temperature and calcium dependence of recovery from the slow component of desensitization, he hypothesized that the D_2 transition was controlled in part by receptor phosphorylation mediated via the enzymatic activity of intracellular protein kinases and/or phosphatases. Paradiso and Brehem (1998) showed that cAMP acting through protein kinase A accelerated recovery from slow desensitization of embryonic muscle nicotinic receptors expressed in *Xenopus* oocytes. In rat chromaffin cells activators of protein kinase C or inhibitors of the phosphatase 2B (calcineurin) accelerated recovery rates from nicotine-induced desensitization (Khiroug et al., 1998).

Our studies indicate that the putative activation of protein kinase C by the phorbol ester PMA or inhibition of protein phosphatase 2B by cyclosporin greatly accelerate the recovery rate from the slow desensitization produced by nicotine. These agents also tended to reduce the overall magnitude of desensitization as well. Similar effects were previously reported in the study of *Xenopus* oocytes transfected with rat $\alpha_4\beta_2$ receptors (Fenster et al., 1999).

The oocyte study similarly reported that 30 min perfusions of 300 nM nicotine desensitized transfected $\alpha_4\beta_2$ nicotinic receptors with an onset composed of fast and slow components having τ values of 1.4 and 17 min, respectively—similar to the 0.6 and 11.3 min values observed here in cortical neurons. The average fractional residual current remaining after a 300 nM nicotine perfusion in the oocyte was virtually identical to that observed in neurons (0.18 vs. 0.20), as was the IC_{50} for nicotine-induced

desensitization (61 nM vs. 51 nM). Thus, the desensitization characteristics of sustained applications of nicotine on $\alpha_4\beta_2$ nicotinic receptors, as well as the effects PMA and cyclosporin A are the same in both native and transfected preparations.

In the neuronal preparation a bi-exponential fit of desensitization onset indicated a much larger fractional proportion of the faster desensitization component at the higher nicotine concentrations tested (100 and 300 nM). In contrast, the proportion of the slower component was generally the same across the range of nicotine concentrations tested. The threshold for nicotinic current activation generally occurs between 100 and 300 nM nicotine (Marszalec et al., 1999). At concentrations below these levels, entry into D_1 desensitization may occur solely by the allosteric stabilization of pre-existing channels already in the desensitized conformation (i.e. $A+D_1 \Leftrightarrow AD_1$. Fenster et al. (1999) have estimated that almost 50% of $\alpha_4\beta_2$ receptors expressed in oocytes equilibrate between R and D_1 in the absence of agonist (see transition Scheme 2). However, the small degree of channel activation incurred at higher concentrations of nicotine may promote additional entry into the D_1 desensitized conformation by the activated receptor-channel pathway (i.e. $A+R \Leftrightarrow AR \Leftrightarrow AD_1$).

The effects of PMA or cyclosporin A on reducing the overall desensitization magnitude and accelerating recovery can be explained by if the equilibrium between D_1 and D_2 is shifted toward the faster recovering D_1 receptor state by nicotinic receptor phosphorylation. Nicotinic receptors contain a large intracellular loop between transmembrane regions M3 and M4 with consensus amino acid sequences capable of undergoing phosphorylation (Swope et al., 1999). Modulation of native nicotinic receptor desensitization by protein kinase or phosphatase activity has been shown for skeletal (Swope et al., 1999; Hoffman et al., 1994), and ganglion-like nicotinic receptors (Vijayaraghavan et al., 1990; Khiroug et al., 1998). Similar effects have also been reported for $\alpha_4\beta_2$ receptors transfected in the aforementioned oocyte preparation (Fenster et al., 1997) and in human embryonic kidney cells (Eilers et al., 1997).

The present study implies that in vivo exposure of α-bungarotoxin-insensitive cortical nicotinic receptors to nicotine concentrations similar to those used here should also undergo a two-stage desensitization sequence. The IC_{50} for nicotine-induced desensitization of the cultured cortical neurons is approximately 50 nM. A tobacco-user's nicotine plasma levels reach sustained plateau levels ranging from 100 to 300 nM (Russell, 1987). Such a continuous exposure should drive most $\alpha_4\beta_2$ receptors into the slowly recovering D_2 state, thereby attenuating much of the endogenous excitatory $\alpha_4\beta_2$-mediated postsynaptic nicotinic signal.

However, such a dampened nicotinic signal can ironically lead to an overall excitatory effect on the brain. This concept arises from reports suggesting that nearly all postsynaptic nicotinic currents in both hippocampus (Frazier et al., 1998; Alkondon et al., 1999; McQuiston and Madison, 1999) and cortex (Xiang et al., 1998; Porter et al., 1999; Alkondon et al., 2000) occur at GABAergic interneurons. Thus, nicotinic receptor desensitization may promote a net disinhibition of some GABAergic inhibitory pathways.

Although no staining techniques were used on the acetylcholine-responsive cortical cells in the present study, their small spindle-shaped morphology clearly distinguishes them from the larger, more numerous pyramidal cells which never respond to applied acetylcholine in the presence of α-bungarotoxin and atropine. Previous studies show that similarly spindle-shaped population of rat cortical interneurons stain heavily for both acetylcholinesterase and GABA synthetic enzymes (Thomas, 1985; Hallanger et al., 1986). We have observed that acetylcholine pulsed onto spontaneously active cultured cortical neurons dramatically increases the frequency of spontaneous inhibitory postsynaptic currents (unpublished data). Thus, the sustained in vivo presence of nicotine encountered with tobacco use could decrease some GABAergic activity via $\alpha_4\beta_2$ desensitization.

The present study demonstrates that the recovery of $\alpha_4\beta_2$-like nicotinic receptors from at least one desensitization state is governed by the activity of endogenous protein kinases and phosphatases. These same enzymes can be modified by drugs of abuse such as alcohol (Stubbs and Slater, 1999; Diamond and Gordon, 1997) or possibly by cellular processes hypothesized as factors underlying psychotic disorders like schizophrenia (Lindow, 2003). Therefore, it may be of interest as a future focus to determine if altered states of nicotine receptor phosphorylation may underlie the comorbidity between alcoholism and cigarette smoking (Rose et al., 2002, 2004).

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