Functional Deactivation of the Major Neuronal Nicotinic Receptor Caused by Nicotine and a Protein Kinase C-Dependent Mechanism

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SUMMARY

The effect of nicotine on the major human neuronal nicotinic receptor (α4β2 subtype) was studied in permanently transfected HEK 293 cells. Prolonged exposure to low concentrations of nicotine (1 μmol/L) increased epibatidine binding but functionally deactivated the nicotinic receptor, abolishing Ca2+ influx in response to an acute nicotine challenge. Deactivation could also be caused by down-regulating protein kinase C (PKC) activity with 0.5 μM phorbol-12,13-dibutyrate or briefly incubating cells with the PKC inhibitor NPC-15437. Recovery from receptor deactivation caused by either nicotine treatment or PKC inhibition occurred slowly (4–6 hr). Reversal of nicotine-induced deactivation was accelerated by the addition of inhibitors of protein phosphatases 2A and 2B. These data suggest a hypothetical mechanism of nicotine-induced deactivation that involves dephosphorylation of nicotinic receptors at PKC phosphorylation sites.

The action of nicotine in the brain is mediated by a family of oligomeric ion channels whose opening is regulated by the binding of the neurotransmitter acetylcholine and drugs such as nicotine. Eleven different mammalian nAChR subunits (α2–9 and β2–4) have been cloned (for reviews, see Refs. 1 and 2), and all the neuronal nicotinic receptors display a pronounced selectivity for Ca2+ relative to Na+. The most abundant receptor is composed of the α4 and β2 subunits with a stoichiometry of 2α3β (6) and is responsible for ∼85% of the high affinity nicotine binding in the brain (6–8).

When nicotine is administered chronically to rats, a ~2-fold up-regulation of high affinity brain nicotinic receptors has been observed (9). This seems to be due to a dramatic decrease in the rate of degradation of the receptor in the cell membrane after nicotine treatment (10). The basis for this change in turnover is obscure. It has also been shown that chronic nicotine exposure leads to what has been termed “functional deactivation” of receptors to distinguish it from short term desensitization (11). Deactivation of neuronal nAChRs was first described and distinguished from receptor desensitization by Simasko et al. (12). The fact that chronic nicotine administration results in an increase in receptor number, coupled with a functional deactivation, suggests a mechanism for the addictive effects of nicotine (13). In this model, withdrawal of nicotine from an individual chronically exposed to the drug would result in reactivation of excess receptors, leading to craving, and prompting a further deactivating dose of the drug (13). Therefore, the relationship between nicotinic receptor number and intrinsic activity is a critical issue. In this report, we show that HEK 293 cells stably expressing the human α4β2 nicotinic receptor subtype, after prolonged exposure to nicotine, display both a dramatic up-regulation of the receptor, together with a functional deactivation that is easily distinguished from simple short term desensitization. This functional deactivation can also be achieved by inhibition of PKC activity in the cells. Furthermore, phosphatase inhibitors increase the rate of recovery from nicotine-induced deactivation of the α4β2 nicotinic receptor. Our data also indicate that the open state conformation per se, rather than calcium ion influx, directs formation of a deactivated receptor structure.

Experimental Procedures

Cloning human nicotinic receptor subunits. A human α4 subunit cDNA probe was generated by RT-PCR of total human brain RNA, with primers based on the rat α4 sequence. The resulting PCR fragment, which contained part of the human α4 sequence spanning nucleotides 755–985 (all numbering is from the ATG initiation

ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; HEK, human embryonic kidney; PKC, protein kinase C; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′,N′-tetraacetic acid; PKA, protein kinase A; PDBu, phorbol-12,13-dibutyrate; RT, reverse transcription; PCR, polymerase chain reaction; PP, protein phosphatase; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
codon) was used to probe a human temporal cortex cDNA library (Stratagene, La Jolla, CA). A cDNA clone was isolated containing nucleotides 382-1076 of the human \( \alpha 4 \) sequence. The cDNA was excised from the AZAP vector as a pBluescript phagemid using the \textit{in vivo} excision protocol. The 5’ region of the \( \alpha 4 \) sequence was obtained by RT-PCR using primers to amplify the sequence from \(-37 \) to \(+525\). This fragment was ligated to the partial \( \alpha 4 \) cDNA in pBluescript, after digestion with XbaI and AatII. The 3’ portion of \( \alpha 4 \) was similarly generated by RT-PCR to amplify the sequence from nucleotides 933-1888. This PCR fragment was ligated to the remainder of the \( \alpha 4 \) cDNA at the unique BspI site. The entire \( \alpha 4 \) cDNA was then subcloned into pCEP4 (InVitrogen, San Diego, CA) for eukaryotic expression.

A human \( \beta 2 \) subunit cDNA was isolated from a thalamus cDNA library (Clontech, Palo Alto, CA) probed with a partial cDNA encoding the rat \( \beta 2 \) subunit. A cDNA clone was isolated, containing nucleotides 100-1468 of the human \( \beta 2 \) subunit. The partial \( \beta 2 \) cDNA was subcloned as an EcoRI fragment from Agt11 into pGEM7zf. The missing 5’ and 3’ ends of the \( \beta 2 \) sequence were obtained by RT-PCR on total human brain RNA. The 5’ end RT-PCR fragment contained the first 135 nucleotides of the \( \beta 2 \) coding region, incorporating a unique NsiI site in the sense primer and including the unique BamHI site found at nucleotide 111. A second RT-PCR fragment was produced that contained a unique PsiI site at nucleotide 1439, and a unique XbaI site at the 3’ end was added. The 5’ and 3’ RT-PCR fragments were ligated to the partial \( \beta 2 \) cDNA clone at the BamHI and PsiI sites, respectively. The full-length \( \beta 2 \) coding region was subcloned as an NsiI/XbaI fragment into pCEP4 to allow for eukaryotic expression. Several clones from both subunit cDNAs derived in this manner were sequenced bidirectionally to ensure that they matched the published sequences for the human \( \alpha 4 \) and \( \beta 2 \) subunit cDNAs (GenBank Accession No. X851458 for \( \alpha 4 \) and X53179 for \( \beta 2 \)).

**Functional assay of transfected cells.** Cells were plated onto rectangular (macrofluorometric assay) or round (microfluorometric assay) glass coverslips suitable for fluorometer applications. The cells were used when they formed a confluent monolayer after \(-48\) hr. The activity of the expressed \( \alpha 4 \beta 2 \) nictinic receptors was assayed by measuring elevations in the intracellular calcium concentration induced by application of nicotine. The method we used is a Fura-2 two-wavelength fluorescence ratio measurement (30). Fura-2 acetyloctamethyl ester (Molecular Probes, Eugene, OR), diluted from a 1 \( \mu \)M stock solution in dimethylsulfoxide with 20% pluronic acid (Molecular Probes), was used at a final concentration of 4 \( \mu \)M in PBS containing 0.1 g/liter CaCl\(_2\), 0.2 g/liter KCl, 8 g/liter NaCl, 0.1 g/liter MgCl\(_2\)-6H\(_2\)O, 0.2 g/liter KH\(_2\)PO\(_4\), and 2.16 g/liter Na\(_2\)HPO\(_4\). The cells were loaded with Fura-2 for 60-90 min at room temperature (21-23\(^\circ\)) before the solution was replaced with fresh PBS.

Macroscopic fluorescence measurements were performed with a Hitachi F-2000 (Tokyo, Japan) fluorescence spectrophotometer. The coverslips were mounted in a quartz cuvette by means of a specially designed holder to keep the sample at the correct angle in the light beam. The cuvettes were filled with 1.8 ml of PBS. Through a hole in the holder, chemicals such as receptor agonists could be injected during data acquisition to measure acute responses. A magnetic stirbar on the bottom of the cuvette ensured rapid mixing of the solutions. The experiments were done at room temperature (21-23\(^\circ\), thermostatically controlled). For the microfluorometric measurements, we used an inverted microscope (Nikon, Tokyo, Japan) with an attached fluorometer (PTI, South Brunswick, NJ). The coverslips were mounted in a perfusion chamber (Warner Instrument Co., Hamden, CT) on the microscope stage. The attached perfusion system with microprocessor-controlled magnetic valves (Automate, Oakland, CA) allowed us to use short exposure times (<15 sec) and with the high flow rate of 11.5 ml/min (volume of perfusion chamber, 150 \( \mu \)l) enabled rapid solution exchanges. For both systems, changes in intracellular calcium ion concentration were measured by determining the ratio of 510 nm light emitted by alternate stimulation with 340 and 380 nm. The background autofluorescence (510 nm) of the unloaded cells was measured and subtracted from the raw signal before calculation of the ratio. We used the changes in the calculated fluorescence ratios (after background subtraction) as indicator for changes in intracellular calcium concentration and did not express the results in absolute values of calcium concentrations. To correct for the variability in the size of the responses from batch to batch and allow comparison of the results, the ratio measurements were normalized. In the dose-response experiment, the values were expressed as percent of the maximal response; in all other experiments, the values were expressed as percent of the control response that is the ratio change after exposure to 10 \( \mu \)M nicotine (otherwise untreated transfected cells).

For long term exposure to nicotine, we replaced the culture medium in the dishes with medium containing the desired nicotine concentration. The cells were kept in an incubator at 37\(^\circ\) for the desired time. Before the assay, they were washed twice in PBS and loaded with Fura-2 at room temperature for 1 hr. When a drug application was terminated and the recovery, or washout, phase began, the coverslips were washed twice in the incubation medium for the next step of the protocol to ensure complete removal of the drug.

**Data analysis.** Data are presented as mean \( \pm \) standard deviation unless otherwise indicated. Where necessary, least-squares curve fitting (Marquardt-Levenberg algorithm) and statistical analysis were performed with SigmaPlot and SigmaStat (Jandel Scientific, Costa Madre, CA). One-way ANOVA with Student-Newman-Keuls test for post hoc comparisons and paired \( t \) test were used as indicated. A value of \( p < 0.05 \) was considered statistically significant.

**Results**

Up-regulation of the \( \alpha 4 \beta 2 \) nAChR in a stably transfected cell line. HEK 293 cells were transfected with cDNAs encoding the human \( \alpha 4 \) and \( \beta 2 \) nAChR subunits. Clonal lines were selected in media containing hygromycin (0.5 mg/ml) and were screened by dot-blot for expression of subunit RNAs. Clones that expressed high levels of both subunit mRNAs were then assayed for the expression of \[^{3}H\]acetylcholine binding activity (14). On this basis, a single clone was selected for further study and was designated 42G. Expression of high affinity \[^{3}H\]epibatidine binding activity in the 42G cells was assayed by means of a plate assay with the anti-\( \beta 2 \) subunit antibody monoclonal antibody 270, essentially according to the protocol used by Peng et al. (10). 42G cells expressed \[^{3}H\]epibatidine binding activity that was almost completely blocked by 1 \( \mu \)M nicotine. However, when these cells were incubated in the continued presence of 1 \( \mu \)M nicotine for 2 days, a considerable increase in epibatidine binding was observed, with a very modest decrease in apparent receptor affinity. The data were fitted to an equation of the form:

\[
\frac{B_{\text{max}}[^{3}H\text{Epi}]}{K_d} + \frac{[^{3}H\text{Epi}]}{K_d}
\]

where \[^{3}H\text{Epi}\] is the concentration of labeled epibatidine, and B is bound epibatidine, from which the Hill coefficient (\( h \)) and apparent \( K_d \) and \( B_{\text{max}} \) values were determined (Fig. 1A). Nicotine treatment generally resulted in a 5–6 fold up-regu-
lation of binding activity (Fig. 1B). This increase in binding is much greater than that reported previously for rat brain or for chick and rat α4β2-expressing cell lines (10). However, a similar up-regulation was reported previously in a cell line transfected with the human α4 and β2 subunits (15). Receptor up-regulation was observed when cells were incubated for 2 days with as little as 10 nM nicotine, with 10 μM nicotine eliciting a maximal effect (Fig. 1B). Two different antagonists at the neuronal nicotinic receptor were tested for their ability to up-regulate epibatidine binding in the 42G cell line. Mecamylamine, an open channel blocker, failed to up-regulate binding (Fig. 1A). In addition, coapplication of mecamylamine and nicotine reduced the amount of up-regulation produced by nicotine (Fig. 1A). Dihydro-β-erythroidine, a competitive antagonist, caused a small increase in B_max values (Fig. 1A). Larger increases in B_max values after higher doses of dihydro-β-erythroidine have been reported by Gopalakrishnan et al. (15).

**Functional assay of α4β2 nicotinic receptors in 42G cells.** The function of nicotinic receptors in 42G cells was examined by measuring the increase in intracellular free calcium after a nicotine stimulus. A representative trace of the fluorescence ratio after a 10 μM nicotine stimulus is shown in Fig. 2A. The trace shows that a stable base-line ratio was maintained for ~80 sec, at which time nicotine was added to the cuvette, resulting in a fluorescence ratio increase of 0.77. In the absence of nicotine, the cells had stable
fluorescence ratios for >20 min. A few samples with unstable base-lines were excluded from the data analysis. Nicotine treatment of 42G cells increased intracellular calcium with an EC50 value of 2.21 ± 0.42 µM (Fig. 2B).

A series of control experiments were conducted to verify that the observed calcium influx was dependent on both the expression of the α4β2 receptor and the presence of extracellular calcium. Nicotine treatment of untransfected HEK 293 cells did not result in changes in intracellular calcium levels (Table 1). In addition, 42G cells showed no response to nico-

...cellular calcium concentration due to calcium ion influx. In contrast, when cells were exposed to nicotine for different periods of time. Because the fluorometric assay we used for most of the experiments did not allow rapid exchanges of the assay solution, we modified the assay by plating 42G cells onto a coverslip and mounting it in a perfusion chamber on a microscope stage. This allowed us to achieve exposure times of ≥5 sec, which was not possible with the assay in the cuvette. Cells were loaded with Fura-2 for 1 hr, followed by incubation with 10 µM nicotine for either 15 sec or 60 min. The cells were then superfused with nicotine-free PBS for 5 min. After this recovery period, the cells were challenged with 10 µM nicotine (15-sec exposure time), and the rise in intracellular calcium was measured. After the washout period, cells preincubated with nicotine for only 15 sec exhibited ~100% of their initial (control) nicotine-stimu-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Medium</th>
<th>Challenge</th>
<th>Mean response ± SD</th>
<th>% of control</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK 293 (untransfected)</td>
<td>PBS</td>
<td>10 µM nicotine</td>
<td>2.7 ± 1.8</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>42G (transfected)</td>
<td>Ca2+-free PBS with 1 mM EGTA</td>
<td>10 µM nicotine</td>
<td>3.5 ± 3.5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>42G (transfected)</td>
<td>PBS</td>
<td>50 mM KCl</td>
<td>0.9 ± 1.8</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

Nicotine has two effects on the responsiveness of α4β2 receptors: a short term desensitization and a much longer functional deactivation. These two effects were distinguished by incubation of 42G cells in the presence of nicotine for different periods of time. Because the fluorometric assay we used for most of the experiments did not allow rapid exchanges of the assay solution, we modified the assay by plating 42G cells onto a coverslip and mounting it in a perfusion chamber on a microscope stage. This allowed us to achieve exposure times of ≥5 sec, which was not possible with the assay in the cuvette. Cells were loaded with Fura-2 for 1 hr, followed by incubation with 10 µM nicotine for either 15 sec or 60 min. The cells were then superfused with nicotine-free PBS for 5 min. After this recovery period, the cells were challenged with 10 µM nicotine (15-sec exposure time), and the rise in intracellular calcium was measured. After the washout period, cells preincubated with nicotine for only 15 sec exhibited ~100% of their initial (control) nicotine-stimu-

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...calculated calcium influx. In contrast, when cells were exposed to nicotine for 1 hr at 37°, washed extensively, and returned to normal culture medium for various times before assaying the response to acute nicotine, ~7 hr of incubation was required to achieve a maximal return of activity. The activity returned to about control level, and this reversal was not due to the synthesis of new receptors because cycloheximide was present throughout the recovery period to block protein syn-

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**Fig. 3.** Desensitization and deactivation of the α4β2 nicotinic receptor after incubation with nicotine. Cells were first exposed to 10 µM nicotine for 15 sec (A) or 1 hr (B). Before the second exposure, cells were allowed to recover for 5 min without any nicotine present. The second exposure had a duration of 15 sec in both cases (B and D). Arrows, start of the nicotine exposure. Scale, applicable to all four tracings. Very little response is seen in the second exposure (D) after the long term exposure (C), whereas the cells recover completely after the short term exposure (A and B). The four tracings were representative of all obtained recordings.

**Fig. 5.** When 42G cells were exposed to 50 µM nicotine for 1 hr at 37°, washed extensively, and returned to normal culture medium for various times before assaying the response to acute nicotine, ~7 hr of incubation was required to achieve a maximal return of activity. The activity returned to about control level, and this reversal was not due to the synthesis of new receptors because cycloheximide was present throughout the recovery period to block protein syn-

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nicotine in the presence or absence of Ca\textsuperscript{2+} gave the same response to an acute nicotine challenge (Table 2). Furthermore, incubation with the noncompetitive antagonist mecamylamine, which is believed to act as an open channel blocker, effectively induced deactivation of the channel. A nondeactivating concentration of nicotine (10 nM) did not have a statistically significant effect on mecamylamine-induced deactivation (Fig. 6).

**PKC activity is necessary for α4β2 receptor function.** Because the α4 subunit contains at least nine serine-specific phosphorylation consensus sites (16), we considered the possibility that receptor deactivation may be regulated by protein kinase activity. In contrast to the α4 subunit, the β2 subunit has no apparent serine-specific phosphorylation consensus sequences. To determine whether the activity of α4β2 receptors is regulated by PKC, we incubated 42G cells for 24 hr in the presence of 0.5 μM PDBu, which is known to down-regulate PKC activity profoundly (17, 18). When PDBU-treated cells were challenged in the calcium flux assay, the response to acute application of nicotine was attenuated by ~80% (Fig. 7A). Activity could be recovered to 58% of control activity after a 6-hr incubation in the absence of PDBU; this presumably reflects the slow recovery of PKC levels to normal. To investigate whether receptors could be modulated more rapidly by direct inhibition of PKC activity, we incubated 42G cells preloaded with Fura-2 with various concentrations of NPC-15437 (Research Biochemicals, Natick, MA), a highly specific inhibitor of PKC, for 5 min before assaying for nicotine-stimulated Ca\textsuperscript{2+} flux (Fig. 7B). As little as 1 μM NPC-15437 depressed nicotine-stimulated calcium influx in 42G cells by ~65%. It should be noted that NPC-15437 did not affect the basal fluorescence of the Fura-2 dye, ruling out an effect of the agent on the assay itself. Increasing the concentration of the inhibitor to 5 μM blocked ~90% of receptor activity. The reported K i value of NPC-15437 for PKC is 19 μM, suggesting that nicotinic receptor activity is sensitive to even partial inhibition of PKC activity. As observed for nicotine-stimulated deactivation, the effect of NPC-15437 reversed slowly over a span of 6–8 hr (data not shown), a rate that compared favorably with reversal after nicotine-induced deactivation (Fig. 5). These data are consistent with a mechanism in which nicotine deactivates receptors by a process that involves receptor dephosphorylation. If this were so, one might expect to see an enhancement in the rate of reversal of nicotine-mediated deactivation by including phosphatase inhibitors in the washout period after a deactivating exposure to nicotine. Indeed, when nicotine-deactivated (1-hr incubation with 50 μM nicotine) 42G cells were incubated in the presence of the phosphatase inhibitors okadaic acid (50 nM) and cypermethrin (50 nM), a significant amount of recovery was observed under conditions in which no recovery was seen

**TABLE 2**

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Medium</th>
<th>Incubation</th>
<th>Challenge</th>
<th>Mean response ± SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>42G (transfected)</td>
<td>Ca\textsuperscript{2+}-free PBS with 1 mM EGTA</td>
<td>50 μM nicotine for 1 hr; 1-hr recovery</td>
<td>10 μM nicotine</td>
<td>2.2 ± 3.4</td>
<td>3</td>
</tr>
<tr>
<td>42G (transfected)</td>
<td>PBS with Ca\textsuperscript{2+}</td>
<td>50 μM nicotine for 1 hr; 1-hr recovery</td>
<td>10 μM nicotine</td>
<td>2.4 ± 4.1</td>
<td>3</td>
</tr>
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</table>
in nicotine-treated cells that received only vehicle in the recovery phase (Fig. 8). Little effect of these agents was seen when they were used separately (data not shown). This is likely due to the presence of multiple PP in mammalian cells. Okadaic acid and cypermethrin inhibit PP-1 and -2A and PP-2B, respectively (19, 20). However, PP-2C is not inhibited by any known agent compatible with viable cells. That inhibition of only two classes of PP accelerated recovery from nicotine-mediated deactivation is, therefore, all the more striking.

Discussion

In the current study, we reconstituted the human α4β2 nicotinic receptor as a functional oligomer in a mammalian cell line. Although the level of expression is modest in the 42G cell line, the ligand binding activity can be determined easily and reliably. Similarly, flux of Ca2+ into the cells can be measured by fluorometric assay when they are challenged by acute application of nicotine. This assay allowed us to probe the function of the receptors and their response to prolonged exposure to nicotine. We found that the acute effect of nicotine on Ca2+ flux was apparent over a range of 0.1–20 μM, which correlates well with data obtained with rat α4β2 receptors in oocytes (21) and human α4β2 receptors in a transfected cell line (15). Similarly, most of the effect of chronic nicotine exposure (2 days) on receptor number, determined by epibatidine binding, was apparent at 1 μM. Hence, in 42G cells there is a reasonable correlation between channel activation and receptor up-regulation.

It has been established broadly that prolonged exposure of α4β2 nicotinic receptors to low concentrations of nicotine results in both a dramatic decrease in the rate of receptor degradation (10) and a long-lasting decrease in the responsiveness of the receptor to agonists (10, 11, 22). Although the effect of nicotine on receptor up-regulation was noted, the current study focuses on the latter problem: the deactivation phenomenon first described by Simasko, in response to carbamylcholine (12), and by Lukas, in response to nicotine (11). Functional deactivation of nAChRs elicited by nicotine does not seem to require the activity of the ion channel per se. This is suggested by the fact that nicotine-mediated deactivation is unhindered by the omission of Ca2+ from the extracellular medium during the incubation and that mecamylamine, an open channel blocker, mimics the effect of nicotine on functional deactivation of the α4β2 receptor.

These data indicate that channels in an open/closed or open/open conformation are subject to some modification that results in a receptor with a decreased ability to pass Ca2+. Full opening of the channel does not seem to be a prerequisite.
for this modification because mecamylamine mimics the deactivating effect of nicotine. Both time of exposure and concentration of the deactivating agent seem to be important factors in the deactivation process, with time being more important than concentration. This is suggested by the decrease of the $E_{C50}$ for receptor deactivation with increasing time of exposure (22). Short incubations with nicotine of <30 sec, on the other hand, fail to deactivate $\alpha 4\beta 2$ receptors even with higher concentrations of the drug, although a more rapidly reversible desensitization is evident. Longer exposures of $\geq$1 hr result in a profound, slowly reversible deactivation of the receptors, arguing for the involvement of some modification of the receptors themselves.

Both agonists and competitive antagonists (e.g., dihydro- $\beta$-erythroidine) cause up-regulation (15). Mecamylamine, on the other hand, a noncompetitive blocker of the channel, failed to up-regulate the $\alpha 4\beta 2$ nicotinic receptor in our model, although mecamylamine-induced up-regulation has been reported at higher concentrations by other investigators (10). We concluded from this that up-regulation and deactivation are likely to be induced by two distinct molecular mechanisms: one described by a competitive interaction and the other by a noncompetitive interaction. A true agonist like nicotine would combine both of these properties and thus both up-regulate and deactivate the receptor.

We have shown that block of PKC activity by either down-regulation or direct inhibition of the enzyme caused deactivation of the $\alpha 4\beta 2$ receptor. This is particularly true of the PKC inhibitor NPC-15437. In contrast, inhibitors of tyrosine kinases or of PKA had no effect on nAChR activity (data not shown). Furthermore, a combination of the phosphatase inhibitors, okadaic acid (PP-1 and -2A) and cypermethrin (PP-2B), caused a dramatic recovery from nicotine-stimulated deactivation under conditions in which no recovery could otherwise be discerned. These data support a specific role for PKC in regulating the activity of the $\alpha 4\beta 2$ nicotinic receptor.

Furthermore, the remarkable correlation between prolonged nicotine exposure and inhibition of PKC activity in inhibiting AChR function, as well as in the reversal from these two types of treatment, must be regarded as strong circumstantial evidence that nicotine-mediated deactivation is brought about (at least partially) by a PKC-dependent mechanism. Although the effect of nicotine on PKC activity has been studied in adrenal chromaffin cells (23), the converse effect of PKC on neuronal nicotinic receptors has not been greatly explored. Further experiments will be needed to establish the exact molecular basis of this phenomenon.

Our experiments suggest a model for the effect of nicotine on the $\alpha 4\beta 2$ receptor. We hypothesize that in the presence of nicotine, $\alpha 4\beta 2$ receptors are rapidly desensitized. The desensitization is likely accompanied by a structural change in the receptor, most importantly in the intracellular loop of the $\alpha 4$ subunit. This in turn might inhibit access of PKC and other kinases, such as PKA, to phosphorylation sites on the receptor. With undiminished efficacy of protein phosphatases, as we propose, this would shift the equilibrium toward the dephosphorylated state. Different phosphatases might be involved in this dephosphorylation cascade, which takes some time to complete, but it is fast compared with the rephosphorylation. Finally, after nicotine-stimulated dephosphorylation process is complete, the receptor enters a stable and functionally deactivated state. Because the cytoplasmic loop of the $\alpha 4$ subunit, but not the $\beta 2$ subunit, is decorated with a multitude of serine phosphorylation consensus sequences, we suggest that dephosphorylation of this region of the $\alpha 4$ subunit leads to a conformational change in the receptor such that ion flux is blocked. One might imagine that charge repulsion induced by phosphorylation causes the large loop region to sit out and away from the channel pore. Conversely, dephosphorylation could cause the loop to hinder ion flux by interacting with pore components on the cytoplasmic face of the receptor. A similar mechanism has been described for the regulation of glycogen synthase activity by phosphorylation (24, 25) and brain Na$^+$ channels by PKA and PKC (26, 27). Effects of phosphorylation on ion channel function are common (25, 26), although the effect in the case of the nicotinic receptor seems to be by far the most profound.

The activity and location of PKC at the nicotinic synapse could, therefore, be an important factor in determining the response of animals to nicotine. It has been shown, for example, that certain strains of mice display altered responses to nicotine (28). It is tempting to speculate that even slight decreases in PKC activity may tip the balance in favor of dephosphorylation and deactivation of $\alpha 4\beta 2$ receptors. However, no experiments seem to have been done that assess the intrinsic activity of nicotinic receptors or PKC activity in the brains of these mice. Activation and deactivation of nicotinic receptors by a phospho/dephospho process may regulate short term sensitivity of synapses to acetylcholine. It would also conceivably allow other neurotransmitter systems to influence the nicotinic receptor response (29). Further work is required, however, to establish the sites of phosphorylation that specifically regulate activation and deactivation of the $\alpha 4\beta 2$ neuronal nicotinic receptor. Such work may explain not only the action of nicotine in addiction but also the plasticity of the cholinergic synapse.
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References


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