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Nicotine-induced up regulation of $\alpha 4\beta 2$ neuronal nicotinic receptors is mediated by the PKC-dependent phosphorylation of $\alpha 4$ subunits

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Abstract

Sustained exposure to nicotine is well known to increase the cell surface density of $\alpha 4\beta 2^*$ neuronal nicotinic receptors both *in vivo* and *in vitro*, but the cellular mechanisms mediating this effect are equivocal. Using a pharmacological approach to investigate the effects of nicotine on receptor subunit expression and phosphorylation in SH-EP1 cells expressing human $\alpha 4$ and $\beta 2$ nicotinic receptor subunits, we have demonstrated that incubation with nicotine for 24 hours increased the expression of immature and mature forms of both $\alpha 4$ and $\beta 2$ subunits in a concentration-dependent manner, and that inhibition of PKC, but not PKA inhibited the nicotine-induced increased expression of subunits. Incubation of cells with nicotine for 24 hours also increased the phosphorylation of immature forms of $\alpha 4$ subunits similar to that induced by activation of either PKC or PKA. When cells were preincubated with nicotine, the PKC-mediated increased phosphorylation was inhibited; the PKA-mediated phosphorylation was unaltered. The phosphopeptide maps for immature $\alpha 4$ subunits following nicotine exposure or PKC activation were identical, and phosphoamino acid analyses indicated phosphorylation on serine residues only. Results indicate that nicotine-induced up regulation of $\alpha 4\beta 2$ neuronal nicotinic receptors involves a PKC-dependent mechanism and likely reflects the ability of nicotine to activate PKC, leading to the phosphorylation of immature $\alpha 4$ subunits, promoting subunit assembly and receptor maturation. Because up regulation of these receptors has been implicated to mediate tolerance, locomotor sensitization and addiction to nicotine, results identify a potential new target for modulating the effects of nicotine on the brain.

Keywords

nicotinic receptor; protein kinase A; protein kinase C; receptor phosphorylation; receptor maturation

It has been known for nearly 20 years that neuronal nicotinic receptors containing $\alpha 4$ and $\beta 2$ subunits exhibit an increased density following sustained exposure to nicotine (Flores et al., 1992). This is a 'universal' phenomenon that occurs in cultured neurons expressing these receptors (Pacheco et al., 2001), following injection of subunit mRNAs into *Xenopus laevis* oocytes (Fenster et al., 1999), and in brain from rats (Schwartz and Kellar, 1983), mice

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(Marks et al., 1993), and humans (Benwell et al., 1988), and is believed to be responsible for many of the effects resulting from sustained nicotine exposure including tolerance, locomotor sensitization, and the addictive process (Tapper et al., 2004). Although studies have demonstrated that sustained nicotine exposure increases the density of $\alpha 4\beta 2^*$ neuronal nicotinic receptors (*indicates that other subunits may be present), the mechanisms involved remain equivocal. Studies have suggested that up regulation may be due to nicotine-induced: 1) increased half-life of plasma membrane associated receptors (Peng et al., 1994; Kuryatov et al., 2005); 2) increased proportion of receptors in a high affinity state at the cell surface (Buisson and Bertrand, 2001; Nelson et al., 2003; Vallejo et al., 2005); and 3) increased receptor subunit assembly and folding, receptor maturation and/or receptor trafficking to the cell membrane (Harkness and Millar, 2002; Nashmi et al., 2003; Darsow et al., 2005; Kuryatov et al., 2005; Sallette et al., 2005; Moroni et al., 2006).

Based on results indicating that nicotine exposure, either *in vitro* or *in vivo*, did not affect subunit steady-state mRNA levels (Peng et al., 1994), initial evidence suggested that increased receptor density must be the consequence of a post-translational event. Rothhut et al. (1996) investigated the role of cAMP-dependent protein kinase (PKA) on the assembly and expression of chicken $\alpha 4\beta 2$ receptors expressed in M10 fibroblasts and demonstrated that incubation of cells with either forskolin or nicotine increased the number of cell surface receptors to a comparable extent. Further, when cells were incubated with both forskolin and nicotine, additive effects were apparent suggesting that the ability of nicotine to increase the number of cell surface receptors could not be attributed to a PKA-mediated event. Similarly, when human embryonic kidney (HEK 293) cells expressing human $\alpha 4\beta 2$ receptors were incubated with nicotine, forskolin or PMA, the latter to activate protein kinase C (PKC), an increased cell surface receptor density was apparent, but simultaneous exposure to nicotine and the kinase activators led to synergistic effects, supporting the idea that nicotine and protein kinase activation work through distinct mechanisms (Gopalakrishnan et al., 1997).

Despite evidence suggesting that the actions of nicotine differ from those of protein kinase activation, recent studies investigating $\alpha 4\beta 2$ receptor assembly and trafficking in both clonal cells and cultured midbrain neurons demonstrated that nicotine incubation increases receptor assembly and functional responses similar to that following PKC activation (Nashmi et al., 2003). Based on these findings, the authors suggested that the ability of nicotine to increase cell surface receptor density may involve a PKC-mediated mechanism involving phosphorylation at key residues on $\alpha 4\beta 2$ receptors perhaps inhibiting recognition of an ER retention sequence, and facilitating surface translocation.

The idea that phosphorylation of nicotinic receptor subunits promotes receptor assembly is supported by studies on both muscle and Torpedo nicotinic receptors demonstrating that the PKA- or PKC-mediated phosphorylation of unassembled γ or δ subunits promotes receptor assembly efficiency and increases the number of cell surface receptors (Green et al., 1991a; 1991b; Ross et al., 1991). Indeed, studies have shown that: 1) both PKA and PKC phosphorylate neuronal $\alpha 4$ subunits (Hsu et al., 1997; Wecker et al., 2001; Viseshakul et al., 1998; Guo and Wecker, 2002; Pacheco et al., 2003); 2) the PKA-mediated phosphorylation of $\alpha 4$ subunits enhances its affinity for the 14-3-3 chaperone protein, resulting in increased expression of $\alpha 4\beta 2$ receptors (Jeanclos et al., 2001); and 3) stimulation of either PKA or PKC increases $\alpha 4\beta 2$ receptor density (Pollock et al., 2009). These findings all support the possibility that the increased density of receptors induced by nicotine involves the PKA- or PKC-mediated phosphorylation of $\alpha 4$ subunits, enhancing the assembly and translocation of $\alpha 4\beta 2^*$ receptors.

To ascertain whether the nicotine-induced increased density of $\alpha 4\beta 2$ receptors could be attributed to a PKA- or PKC-dependent mechanism, human clonal epithelial cells stably

transfected with human $\alpha 4\beta 2$ receptors (SH-EP1-h $\alpha 4\beta 2$ cells) were exposed to nicotine in the presence or absence of compounds that modify the activity of PKA and PKC, and receptor subunit expression and phosphorylation were determined.

EXPERIMENTAL PROCEDURES

Materials

The following antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for immunoblotting: a primary polyclonal anti- $\alpha 4$ antibody (H-133, sc-5591); a primary polyclonal anti- $\beta 2$ antibody (H-92, sc-11372); a secondary goat anti-rabbit-HRP antibody (sc-2030); a goat anti-human actin primary antibody (C-11, sc-1615); and a donkey anti-goat-HRP secondary antibody (sc-2033). For immunoprecipitation experiments, the anti- $\alpha 4$ monoclonal antibody mAb299 was used (MRT-613R, Covance Research Products, Denver, PA) as it has been shown that this antibody binds to both the lower and higher molecular weight species of the $\alpha 4$ subunit (Pollock et al., 2007; 2009). ^{32}Pi was purchased from Perkin-Elmer Life and Analytical Sciences Inc. (Boston, MA), X-ray film from Eastman Kodak Co. (Rochester, NY), enhanced chemiluminescence (ECL) reagents from Amersham-Biosciences Corporation (Piscataway, NJ), and polyvinylidene difluoride (PVDF) membranes (Immobilon-P) from the Millipore Corporation (Billerica, MA). DMEM, penicillin, streptomycin, L-glutamine, and horse serum were purchased from Invitrogen (Carlsbad, CA), Fetal Clone II from HyClone (Logan, UT), and forskolin, PDBu and RO-31-8220 from Calbiochem (San Diego, CA). Basic electrophoresis chemicals and protein markers were obtained from Bio-Rad Laboratories (Hercules, CA), and protein G and additional molecular weight markers were purchased from Sigma-Aldrich (St. Louis, MO). Thin-layer chromatography (TLC) plates were obtained from VWR International Inc. (Bristol, CT) and sequencing grade trypsin was purchased from Roche Diagnostics Corporation (Indianapolis, IN).

Expression of mature and immature $\alpha 4$ and $\beta 2$ subunit proteins

Human clonal SH-EP1-h $\alpha 4\beta 2$ cells (kindly provided by Dr. Ron Lukas, Barrow Neurological Institute, Phoenix, AZ) were used for all studies. Cells were grown and maintained at 37°C (with 5% CO_2) in DMEM (high glucose) containing 5% fetal clone II, 10% horse serum, 1 mM sodium pyruvate, 8 mM L-glutamine, 100 U/ml penicillin, 100 μg /ml streptomycin, 0.05 mg/ml amphotericin B, 0.5 mg/ml zeocin and 0.4 mg/ml hygromycin B, the latter two for selection of the receptor. Cells were kept in continuous culture, fed every 2–3 days, and were grown to 80–85% confluence in 60 mm dishes.

For studies investigating the concentration-dependent effects of nicotine on the expression of $\alpha 4$ and $\beta 2$ subunits, cells were washed and incubated with nicotine (0–1000 nM) for 24 hours. For studies investigating the effects of PKC inhibition on the up regulation of $\alpha 4$ and $\beta 2$ subunit protein, cells were incubated in the absence or presence of the PKC inhibitor RO-31-8220 (0.1 μM) for 30 minutes, followed by the addition of forskolin (10 mM in 0.1% DMSO), phorbol 12,13-dibutyrate (PDBu, 200 nM in 0.1% DMSO), or nicotine (50 nM) and incubated for 24 hours. Following incubation, whole cell lysates were prepared by washing the cells (5 x) with ice-cold phosphate buffered saline (PBS) followed by incubation for 30 minutes at 4°C in 400 ml of potassium phosphate (200 mM) homogenization/lysis buffer (pH 7.4) containing 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM β -glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4 , 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin A, 10 μg /ml leupeptin, 10 U/ml aprotinin, and 2% Triton X-100. The plates were scraped and samples were transferred into microfuge tubes and triturated by mechanical disruption (20 strokes) using a 1 ml syringe with a 26-gauge needle; samples were further solubilized for 30 minutes at 4°C on a rotator. Samples

were centrifuged at $20,000 \times g$ for 20 minutes, and the supernatants were withdrawn and stored at -80°C . For immunoblotting, samples were thawed on ice, resuspended in an equal volume of 2X Laemmli buffer (Laemmli, 1970) and boiled for 5 minutes. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% acrylamide gels and transferred to PVDF membranes. The membranes were blocked with Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.05% Tween-20 for 2 hours at 24°C . To determine the relative quantities of both $\alpha 4$ and $\beta 2$ subunit protein, the membranes were cut horizontally just above the 59 kDa prestained marker between the $\alpha 4$ and $\beta 2$ protein bands, and the top halves of the membranes were probed for $\alpha 4$ subunit protein and the bottom halves for $\beta 2$ subunit protein. The relative quantity of $\alpha 4$ subunit protein was determined using a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 342–474 of the human $\alpha 4$ subunit (H-133, 200 mg/ml used at a 1:2000 dilution); $\beta 2$ subunit protein was determined using a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 342–433 of the human $\beta 2$ subunit (H-92, 200 mg/ml used at a 1:2000 dilution). Membranes were incubated with their respective antibodies in TBS containing 5% non-fat dry milk and 0.05% Tween-20 for 1 hour at 24°C . The membranes were washed and incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (400 $\mu\text{g}/\text{ml}$ used at 1:2000 dilution) for 30 minutes, and the $\alpha 4$ and $\beta 2$ signals were visualized using ECL. Blots were also probed for β -actin to normalize among samples. Immunoblots were analyzed by densitometry using a Bio-Rad Imaging Densitometer with Multi-Analyst software (Bio-Rad Laboratories, Hercules, CA). Sigma “Precision Plus” Kaleidoscope protein markers were used for reference.

^{32}P i labeling and $\alpha 4$ subunit phosphorylation

SH-EP1-h $\alpha 4\beta 2$ cells were grown and maintained to 80–85% confluence as above. To label endogenous ATP stores, the cells were washed 3 times with 2 ml phosphate-free DMEM containing 8 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, and incubated at 37°C in 5% CO_2 for 4 hr in phosphate-free media containing 0.5 mCi/ml ^{32}P i (1 mCi/plate for phosphorylation experiments and 2 mCi/plate for phosphopeptide mapping).

For experiments investigating the effects of nicotine on $\alpha 4$ subunit phosphorylation, cells were incubated in DMEM in the absence or presence of 50 nM nicotine for 20 hours prior to the 4 hour labeling period. Following the 20 hour incubation period in the absence of ^{32}P i, cells were washed and incubated with ^{32}P i as above for 4 hours in the absence or presence of 50 nM nicotine, the latter to control for any potential reversible nicotine-induced alterations during this time. For experiments investigating the effects of nicotine on the PKA- and PKC-dependent phosphorylation of $\alpha 4$ subunits, cells were incubated in the absence or presence of 50 nM nicotine for 24 hours in the absence of ^{32}P i, washed, and incubated with ^{32}P i for 4 hours. Forskolin (10 μM) or PDBu (100 nM) in 0.1% DMSO was added during the final 15 or 30 minutes of the 4 hour labeling period, respectively. These concentrations and times of incubation were chosen based on studies indicating that these parameters led to maximal kinase activation and did not alter the amount of ^{32}P i taken up by the cells during the 4 hour labeling period.

Following incubation, cells were washed 5 $\times$ with ice-cold phosphate buffered saline (PBS), followed by the addition of 500 μl aliquots of homogenization buffer (200 mM potassium phosphate, 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM β -glycerophosphate, 50 mM NaF, 1 mM NaVO_3 , 0.1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 U/ml aprotinin). The plates were scraped, the cells were transferred to 1.5 ml centrifuge tubes and triturated by mechanical disruption as above. Samples were centrifuged at $500 \times g$ for 5 min at 4°C , and the supernatants were withdrawn and

centrifuged at $20,000 \times g$ for 30 min at 4°C . The resultant membrane pellets were resuspended in homogenization buffer containing 2% Triton X-100, solubilized for 30 min at 4°C , and centrifuged at $230,000 \times g$ for 10 min at 4°C . The supernatants containing the ^{32}P -labeled solubilized proteins were withdrawn and $\alpha 4$ subunit protein was immunoprecipitated from the detergent extracts by rotating overnight at 4°C with $5 \mu\text{g}$ of mAb 299 pre-coupled to protein-G sepharose beads. This monoclonal antibody preparation has been shown to bind to both the predominant immature 71–75 kDa $\alpha 4$ protein, as well as the mature 80–85 kDa protein (Pollock et al., 2007; 2009). The bead/antibody/receptor complex was washed 5 times with 100 ml of final storage buffer (50 mM MOPS, 1 mM Na_2EDTA , 1 mM EGTA, pH 7.2 and 0.2% Triton-X 100), resuspended in $20 \mu\text{l}$ Laemmli buffer and boiled for 5 minutes. Following centrifugation at $2,000 \times g$ for 2 minutes, the proteins from the supernatant were separated on SDS-PAGE gels and transferred to PVDF membranes. The membranes were exposed to Kodak XAR film in the presence of intensifying screens for 2–5 hours at -80°C to visualize ^{32}P -labeled $\alpha 4$ subunit protein by autoradiography prior to immunoblotting. The relative quantity of $\alpha 4$ subunit protein was determined by immunoblot analysis using H-133 as above. Autoradiographs and immunoblots were quantitated by densitometry and the relative phosphorylation was expressed as the ratio of the ^{32}P signal from the autoradiograph divided by the protein level from the immunoblot.

Phosphopeptide mapping and phosphoamino acid analyses

Two-dimensional (2-D) phosphopeptide mapping and phosphoamino acid analysis of the immunoprecipitated ^{32}P -labeled $\alpha 4$ subunits were performed using standard techniques as described in detail (Pollock et al., 2007; 2009). Briefly, PVDF membrane bands containing the ^{32}P -labeled $\alpha 4$ protein were excised and processed for either 2-D phosphopeptide mapping or phosphoamino acid analysis. For the former, samples were digested with trypsin, spotted onto cellulose TLC plates, and subjected to electrophoresis in the first dimension and ascending chromatography in the second dimension. Tryptic phosphopeptide patterns were visualized by exposing the plates to XAR film with intensifying screens. Due to large differences in the amount of phosphorylated material obtained under different experimental conditions, within each set of experiments, samples containing equal amounts of radioactivity (1000 cpm) and not equal amounts of protein were analyzed.

For phosphoamino acid analysis, samples were digested with acid and resuspended in buffer containing the standards phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y). Samples containing 100 cpm were spotted onto cellulose TLC plates, and were subjected to electrophoresis at 1.5 kV for 20 min in pH 1.9 buffer in the horizontal direction, and at 1.6 kV for 16 min in pH 3.5 buffer in the vertical dimension. The migration of the phosphoamino acid standards was visualized by spraying the TLC plates with 0.25% (w/v) ninhydrin followed by exposing the plates to Kodak XAR film with intensifying screens for 1–6 weeks at -80°C . The kinase-dependent phosphorylation of serine, threonine, or tyrosine was identified based on the co-migration of the ^{32}P -labeled residues with the stained phosphoamino acid standards.

Statistical Analyses

Grouped data are presented as the mean + S.E.M. For concentration-response curves, data were analyzed using GraphPad PRISM (San Diego, CA). For all other experiments, data were analyzed by analysis of variance (ANOVA); in those instances where significant ($p < 0.05$) main effects were noted, individual group differences were determined by Newman-Keuls test. A level of $p < 0.05$ was accepted as evidence of a statistically significant effect.

RESULTS

Nicotine increases receptor subunit expression by a PKC-dependent mechanism

The first series of experiments characterized the concentration-dependent effects of nicotine on the expression of $\alpha 4$ and $\beta 2$ subunits associated with immature ($\alpha 4_i$ and $\beta 2_i$) and mature ($\alpha 4_m$ and $\beta 2_m$) complexes, the former representing subunits present in complexes located primarily in the endoplasmic reticulum, and the latter representing a mixture of high mannose and complex oligosaccharides likely corresponding to subunits isolated from fully processed mature forms of the receptor associated with the trans-Golgi and plasma membrane (Sallette et al., 2005; Pollock et al., 2009). Results demonstrate that both immature and mature species of $\alpha 4$ and $\beta 2$ were apparent in the cell lysates (Figure 1a) with $\alpha 4_i$ (71–75 kDa) and $\beta 2_i$ (47 kDa) predominant and representing approximately 3–4 times the amount of $\alpha 4_m$ (80–85 kDa) and $\beta 2_m$ (50 kDa) when normalized to β -actin (Figure 1b), in agreement with prior studies (Harkness and Millar, 2002; Sallette et al., 2005; Pollock et al., 2007). Following incubation of cells with nicotine for 24 hours, concentration-dependent increases in the amount of both species of $\alpha 4$ and $\beta 2$ subunits were apparent with a maximal effect following 24 hours incubation with 300 nM nicotine. When data were expressed as percent of corresponding controls, significant ($p < 0.05$) increases were apparent at all concentrations of nicotine tested for all subunit species (Figure 1c). Further, at the lowest concentrations of nicotine (50 and 100 nM), the relative increase in mature and immature forms of $\alpha 4$ subunits did not differ from each other, but as the concentration of nicotine increased, a greater effect was apparent on the expression of the mature form of $\alpha 4$, with a significant ($p < 0.05$) increase in the expression of $\alpha 4_m$ relative to $\alpha 4_i$ following incubation with 300 and 1000 nM nicotine, consistent with the action of nicotine as a maturation enhancer, stabilizing $\alpha 4\beta 2$ complexes and increasing their surface expression (Sallette et al., 2005).

Based on data suggesting that the ability of nicotine to increase cell surface density may involve the PKC-dependent phosphorylation of $\alpha 4\beta 2$ receptors facilitating surface translocation (Nashmi et al., 2003), experiments investigated whether inhibition of PKC could prevent the nicotine-induced up regulation. To accomplish this, cells were incubated for 24 hours with 10 mM forskolin, 200 nM PDBu or 50 nM nicotine, conditions that have been shown to increase the number of membrane-associated $\alpha 4\beta 2$ receptors in these cells (Pacheco et al., 2003; Pollock et al., 2009), in the absence or presence of the PKC inhibitor RO-31-8220 (0.1 μ M). Following incubation, whole cell lysates were prepared, proteins were separated, and immunoblots were probed for the expression of the mature and immature species of both $\alpha 4$ and $\beta 2$ subunit proteins. A representative immunoblot and grouped data are shown in Figure 2. Results indicate that following incubation of cells with forskolin, PDBu or nicotine, the relative amounts of both species of $\alpha 4$ and $\beta 2$ subunits increased significantly ($p < 0.05$). Further, the presence of the PKC inhibitor RO-31-8220 totally prevented the nicotine- and PDBu-induced increases in the immature and mature forms of both $\alpha 4$ and $\beta 2$ subunits. The specificity of RO-31-8220 as a PKC inhibitor was confirmed by evidence that this compound had no effect on forskolin-induced increases. Further, incubation with RO-31-8220 by itself had no effect on subunit abundance. These results support the idea that the ability of nicotine to up regulate $\alpha 4\beta 2$ receptors is mediated through a PKC-dependent process, perhaps involving the phosphorylation of $\alpha 4$ subunit

Nicotine phosphorylates $\alpha 4_i$ subunit expression by a PKC-dependent mechanism

To investigate the possibility that the effects of nicotine are mediated by PKC-dependent phosphorylation, the effects of nicotine on the phosphorylation of $\alpha 4$ subunits were investigated. To accomplish this, cells were incubated in the absence or presence of 50 nM nicotine for 20 hours prior to 4 hours incubation with 32 Pi to label ATP stores. To control

for the possibility that $\alpha 4$ subunits were phosphorylated during the 20 hour incubation with nicotine, followed by dephosphorylation during the 4 hour labeling period, the labeling incubation was conducted both in the presence and absence of 50 nM nicotine. Following incubation, $\alpha 4$ subunits were immunoprecipitated from a membrane preparation, and subunit phosphorylation of the most abundant $\alpha 4$ species ($\alpha 4_i$) was determined. A representative autoradiograph and immunoblot and grouped data are shown in Figure 3. Results indicate that incubation of cells with 50 nM nicotine for 20 hours led to an 88% increase ($p < 0.05$) in the phosphorylation of $\alpha 4_i$ (condition [C]). Results also demonstrate that the presence of nicotine only during the 4 hour labeling period (condition [B]) led to a 23% increase ($p < 0.05$) in subunit labeling, and that when nicotine was present during both the 20 hour incubation and the 4 hour labeling period (condition [D]), the increased phosphorylation of $\alpha 4_i$ did not differ significantly from that achieved when nicotine was absent during the labeling period. Thus, results indicate that prolonged exposure of cells to 50 nM nicotine leads to increased $\alpha 4_i$ subunit phosphorylation in a time-dependent manner. Further, because the increased phosphorylation following 20–24 hours incubation of cells with nicotine was significantly ($p < 0.05$) greater than the increase achieved when nicotine was present only during the labeling period, this effect of nicotine could not be attributed to an action of nicotine to increase $^{32}\text{P}_i$ uptake by cells.

Based on studies indicating that nicotine activates PKC (Messing et al., 1989; Tuominen et al., 1992; Koide et al., 2005), in concert with evidence of increased activity of PKA in postmortem brain from cigarette smokers (Hope et al., 2007), it was possible that the nicotine-induced increased $\alpha 4_i$ subunit phosphorylation involved activation of one or both of these kinases. Indeed, studies in our laboratory have demonstrated that activation of PKA by forskolin or PKC by PDBu increases $\alpha 4$ subunit phosphorylation (Pacheco et al., 2003; Pollock et al., 2007; 2009). Thus, to determine whether the ability of nicotine to enhance $\alpha 4$ subunit phosphorylation was due to the activation of PKA or PKC, cells were incubated with 50 nM nicotine for 24 hours in the absence of $^{32}\text{P}_i$, washed and incubated for 4 hours with $^{32}\text{P}_i$. Forskolin (10 μM) or PDBu (200 nM) were added during the final 15 or 30 minutes of the labeling period, respectively [concentrations and times of incubation that produce maximal kinase activation without altering the total radioactivity taken up by cells during the 4 hour labeling period and lead to increased $\alpha 4_i$ subunit phosphorylation (Pacheco et al., 2003; Pollock et al., 2007; 2009)], and the phosphorylation of both the mature and immature species of the $\alpha 4$ subunit was determined. It was predicted that if nicotine phosphorylated $\alpha 4$ by activation of either or both of the kinases, then following exposure of the cells to nicotine, incubation with forskolin or PDBu should be without effect. A representative autoradiograph and immunoblot and grouped data are shown in Figure 4. In agreement with prior studies (Pollock et al., 2009), results indicate that both forskolin and PDBu increased the relative phosphorylation of the immature species of $\alpha 4$ subunits significantly ($p < 0.05$), and only PDBu increased phosphorylation of the mature species ($p < 0.05$). Further, when cells were incubated with 50 nM of nicotine for 24 hours prior to exposure to the kinase activators, the PDBu-induced phosphorylation of $\alpha 4_i$ was prevented, and phosphorylation did not differ from samples incubated with nicotine alone. There was no effect of nicotine incubation on the forskolin-induced phosphorylation of $\alpha 4_i$. In addition, nicotine incubation did not affect the phosphorylation of the mature species of $\alpha 4$, and did not alter the PDBu-induced phosphorylation of this form of the subunit. These results indicate that nicotine and PDBu share a common mechanism to activate PKC, leading to the phosphorylation of specific substrate sites on $\alpha 4_i$.

To test this possibility, cells were labeled with $^{32}\text{P}_i$ for 4 hours in absence or presence of 50 nM nicotine or 200 nM PDBu during the final 30 minutes of incubation. Proteins were immunoprecipitated from detergent-solubilized membranes with mAb299 and separated by SDS-PAGE gel electrophoresis. The labeled immature $\alpha 4$ subunit protein was excised from

the membranes, digested with trypsin and subjected to 2-D phosphopeptide mapping and phosphoamino acid analysis. Results (Figure 5) indicate that samples incubated with nicotine yielded 2-D phosphopeptide maps for $\alpha 4_i$ that were identical to those following incubation with PDBu. Of particular importance is the increased phosphorylation of phosphopeptide fragments contained within clusters C5 and C6, which represent residues phosphorylated only on immature $\alpha 4$ subunits following stimulation of PKC (Pollock et al., 2009). It is also important to note that under control conditions, peptide fragments contained within cluster C5 were not phosphorylated appreciably and those within C6 were not phosphorylated at all. Further, these fragments are phosphorylated only on immature and not mature $\alpha 4$ subunits, and are not phosphorylated upon stimulation of PKA with forskolin (Pollock et al., 2007;2009). Phosphoamino acid analysis indicates that only serine residues are phosphorylated under any condition examined. These data indicate that nicotine and PKC phosphorylate two common sites on immature $\alpha 4$ subunits and strongly suggest that nicotine may have its actions by stimulating PKC.

DISCUSSION

The objective of the current experiments was to determine whether the well documented nicotine-induced up regulation of $\alpha 4\beta 2$ receptors was due to a PKA- or PKC-dependent mechanism involving the phosphorylation of $\alpha 4$ neuronal nicotinic receptor subunits. Results indicate that: 1) incubation of SH-EP1- $\alpha 4\beta 2$ cells with nicotine for 24 hours led to a concentration-dependent increase in the expression of immature and mature forms of both $\alpha 4$ and $\beta 2$ subunits similar to that induced by incubation of the cells with either forskolin or PDBu to activate PKA or PKC, respectively; 2) the nicotine- and PDBu-induced increased expression of $\alpha 4$ and $\beta 2$ subunits was prevented by concurrent incubation with the PKC inhibitor RO-31-8220; 3) incubation of cells with nicotine for 24 hours led to increased phosphorylation of immature forms of $\alpha 4$ subunits similar, albeit not as robust as that induced by incubation of the cells with either forskolin or PDBu; 4) incubation of cells with nicotine for 24 hours prevented the PDBu-induced phosphorylation of immature, but not mature $\alpha 4$ subunits and did not affect the forskolin-induced phosphorylation of the former; and 5) both nicotine and PDBu phosphorylate only serine residues on immature $\alpha 4$ subunits and lead to the generation of virtually identical phosphopeptide fragments from $\alpha 4_i$. These results indicate that nicotine-induced up regulation of $\alpha 4\beta 2$ neuronal nicotinic receptors involves a PKC-dependent mechanism and likely reflects the ability of nicotine to activate PKC, leading to the phosphorylation of immature $\alpha 4$ subunits, enhancing neuronal nicotinic receptor subunit assembly and receptor maturation.

More than 20 years ago, TerBush and Holz (1986) reported that nicotinic agonists activated PKC in bovine adrenal chromaffin cells, and since that time, studies have replicated this finding (Tuominen et al., 1992), and have shown that nicotinic receptor stimulation increases the translocation/activation of PKC using several cell types including PC12 cells (Messing et al., 1989), smooth muscle cells (Koide et al., 2005), and human umbilical vein endothelial cells (Ueno et al., 2006). This finding can now be extended to include SH-EP1- $\alpha 4\beta 2$ cells as evidenced by: 1) the generation of identical 2-D phosphopeptide maps and the enhanced phosphorylation of two common sites on immature $\alpha 4$ subunits following incubation of cells with either nicotine or PDBu; 2) inhibition of nicotine- and PDBu-induced up regulation of $\alpha 4$ and $\beta 2$ subunits by the PKC inhibitor RO-31-8220; and 3) prevention of PDBu-induced $\alpha 4$ subunit phosphorylation by prior exposure of cells to nicotine. Measures of PKC activity in cells (data not shown) indicated that nicotine did not activate PKC directly. It is likely that nicotine, by activating cell surface receptors, increases intracellular Ca^{2+} concentrations, decreasing the affinity of the pseudosubstrate domain for the PKC catalytic site leading to protein unfolding and enzyme activation, directing the enzyme to the endoplasmic reticulum via the RACK anchoring protein. This idea is

consistent with evidence that nicotine has a 'maturing enhancing' effect on immature $\alpha 4\beta 2$ complexes and may 'rescue' misfolded oligomers that otherwise may be degraded (Sallette et al., 2005), perhaps by stabilizing conformations through PKC-mediated subunit phosphorylation. It is also possible that this mechanism represents the previously undefined 'second messenger signaling system' whereby the activation of cell surface receptors by nicotine increases the number of receptors transiting from the endoplasmic reticulum to the cell membrane, as suggested by Darsow et al. (2005).

Until recently, most studies using immunoblot analyses to investigate alterations in the ability of nicotine to increase the density of $\alpha 4\beta 2^*$ receptors have focused on the predominant lower molecular weight form of the $\alpha 4$ subunit. Sallette et al. (2005) demonstrated that both $\alpha 4$ and $\beta 2$ subunits exist in at least two forms that migrate as different molecular weight proteins on Western immunoblots, a more abundant lower molecular weight form representing immature species present in the endoplasmic reticulum, and a less abundant, higher molecular weight form representing mature species complexed with high mannose and oligosaccharides and associated primarily in the plasma membrane. Further, these authors demonstrated that nicotine acts intracellularly to enhance subunit complex oligomerization. Studies in our laboratory (Pollock et al., 2009) confirmed and extended these findings and identified 3 forms of the $\alpha 4$ subunit upon immunoprecipitation with mAb290, a high molecular weight species at 80–85 kDa, a predominant lower species at 70–75 kDa, and a minor protein band at 66 kDa, the former two corresponding to the mature and immature species and the latter likely corresponding to a fully deglycosylated degradation product as identified by Sallette et al. (2005). Further, we demonstrated that the mature species of $\alpha 4$ was not demonstrable in samples immunoprecipitated with mAb299 from Sigma-Aldrich Co., but was present in preparations immunoprecipitated with mAb299 obtained from Covance Inc. (Pollock et al., 2007; 2009). Results from the present study demonstrate that both species of $\alpha 4$ subunit protein can be detected in whole cell lysates (Figure 1), and immunoprecipitation is not necessary. Thus, using whole cell lysates for measures of subunit protein abundance eliminates apparent discrepancies between measures of receptor densities by radioligand binding versus immunoprecipitation using conformation-dependent antibodies and cell surface biotinylation studies (Kuryatov et al., 2005).

Based on evidence that incubation of cells with nicotine for 24 hours increased the expression of mature forms of the subunits to a greater extent than immature forms, results suggest that nicotine promotes maturation of the receptor complex. Further, within the limitations of these experiments, no significant changes in the ratio of $\alpha 4_m:\beta 2_m$ were observed following nicotine exposure suggesting that nicotine did not alter the stoichiometry of mature receptors. Thus, results do not support the idea that nicotine shifts the equilibrium between high- and low-affinity states of the receptor or that nicotine promotes an expression/maturation pathway to favor one particular stoichiometry over the other, i.e., high affinity $(\alpha 4)_2(\beta 2)_3$ versus low affinity $(\alpha 4)_3(\beta 2)_2$ receptors (Nelson et al., 2003; Vallejo et al., 2005). Rather, results support the idea that nicotine acts on an immature population of subunits/oligomers to facilitate the conversion of these precursors into mature species with higher metabolic stability, leading to up regulation of the mature receptor (Sallette et al. 2005). Further, evidence supports the idea that this process is mediated by activation of PKC, as suggested but not investigated directly by Nashmi et al. (2003).

It is tempting to speculate that activation of PKC by nicotine and phosphorylation of $\alpha 4$ subunits promotes subunit or oligomeric complex stability by promoting interaction with chaperone proteins. Studies have demonstrated that phosphorylation of unassembled $\alpha 4$ subunits by PKA increases association with the 14-3-3 chaperone protein, increasing subunit steady-state levels (Jeanclos et al., 2001). The idea of a similar process involving the PKC-

mediated phosphorylation of $\alpha 4$ subunits either in the free form or as immature $\alpha 4\beta 2$ complexes has not yet been investigated. Although the assembly and maturation of many types of nicotinic receptors depends on interactions with endoplasmic reticulum chaperone proteins (Millar and Harkness, 2008), little is known about the role of phosphorylation in mediating these protein:protein interactions. One protein of interest is the RIC-3 chaperone protein as recent studies have demonstrated a positive correlation between the expression of this chaperone protein and that of $\alpha 4$ and $\beta 2$ subunits in human brain (Severance and Yolken et al., 2007) and evidence that RIC-3 enhances the expression of $\alpha 4\beta 2$ receptors in human kidney tsA201 cells (Lansdell et al., 2005).

In sum, results support the idea that nicotine acts as a pharmacological chaperone (Kuryatov et al., 2005) to increase the assembly and maturation of $\alpha 4\beta 2^*$ neuronal nicotinic receptors, and indicate that its action is mediated by activation of PKC, resulting in the phosphorylation of immature $\alpha 4$ subunits on serine residues and promoting receptor assembly and maturation. Further, because PKC phosphorylation sites on $\alpha 4$ subunits are located within the M3/M4 cytoplasmic domain of the protein (Wecker et al., 2001; Pollock et al., 2007), results support evidence that this large intracellular loop has a major influence on receptor assembly and ultimately, function. Additional studies will undoubtedly delineate the role of $\alpha 4$ subunit phosphorylation in mediating protein:protein interactions required for the nicotine-induced increased expression of cell surface $\alpha 4\beta 2^*$ receptors, identifying new targets for the pharmacological modulation of these receptors, perhaps leading to new approaches to the treatment of addiction.

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Abbreviations

$\alpha 4_i$	immature $\alpha 4$ subunits
$\alpha 4_m$	mature $\alpha 4$ subunits
$\beta 2_i$	immature $\beta 2$ subunits
$\beta 2_m$	mature $\beta 2$ subunits
ECL	enhanced chemiluminescence
HRP	horseradish peroxidase
PBS	phosphate buffered saline
PDBu	phorbol 12,13-dibutyrate
PVDF	polyvinylidene difluoride
Pi	inorganic phosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TLC	thin-layer chromatography
2-D	two-dimensional

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Fig. 1.

Effects of nicotine on the expression of $\alpha 4$ and $\beta 2$ subunit protein. SH-EP1-h $\alpha 4\beta 2$ cells were incubated with 0–1000 nM nicotine for 24 hours. Whole cell lysates were prepared, proteins were separated by SDA-PAGE gel electrophoresis and transferred to PVDF membranes. Membranes were cut horizontally just above the 59 kDa prestained marker between the $\alpha 4$ and $\beta 2$ protein bands. The top half of the membranes was probed for $\alpha 4$ subunit protein with the polyclonal anti- $\alpha 4$ antibody H-133, and the bottom halves were probed for $\beta 2$ subunit protein with the polyclonal anti- $\beta 2$ antibody H-92; β -actin was probed with the polyclonal antibody C-11. A representative immunoblot identifying mature ($\alpha 4_m$ and $\beta 2_m$) and immature ($\alpha 4_i$ and $\beta 2_i$) species of the subunits and β -actin with molecular weight standards are shown. The bar graphs represent group mean values +

S.E.M. from 5 experiments. Subunit expression was determined by normalizing the subunit protein signals to β -actin and results expressed relative to controls to account for variations in protein expression in the cells. *Significant difference from controls, $p<0.05$; ‡, significant difference between subunit species, $p<0.05$.

Fig. 2.

Effects of nicotine and protein kinase activation and inhibition on the expression of $\alpha 4$ and $\beta 2$ subunit protein. SH-EP1-h $\alpha 4\beta 2$ cells were incubated in the absence or presence of the PKC inhibitor RO-31-8220 (0.1 μ M) for 30 minutes, followed by the addition of forskolin (10 μ M in 0.1% DMSO), PDBu (200 nM in 0.1% DMSO) or nicotine (50 nM) and incubated for an additional 24 hours. Whole cell lysates were prepared, and samples processed as for Figure 1. A representative immunoblot and bar graphs representing group mean values + S.E.M. from 5 experiments are shown. Subunit expression was determined by normalizing the subunit protein signals to β -actin and results expressed relative to controls to account for variations in protein expression in the cells. *Significant difference from controls, $p < 0.05$; ‡, significant difference between corresponding group values determined in the absence of RO-31-8220, $p < 0.05$.

Fig. 3.

Effects of nicotine on the phosphorylation of $\alpha 4_i$ subunits. SH-EP1-h $\alpha 4\beta$ 2 cells were incubated in the absence or presence of 50 nM nicotine for 20 hours, washed, and incubated for an additional 4 hours with ^{32}P i in the absence or presence of 50 nM nicotine. The $\alpha 4$ subunits were immunoprecipitated from detergent-solubilized membranes with mAb299. Proteins were separated by SDS-PAGE gel electrophoresis, and samples subjected to autoradiography and immunoblotting. A representative autoradiograph and immunoblot are shown for: [A], a single sample incubated in the absence of nicotine for 20 hours followed by incubation with ^{32}P i; [B], lysates from 2 samples incubated for 20 hours in the absence of nicotine followed by incubation for 4 hours with ^{32}P i in the presence of nicotine; [C], lysates

from 2 samples incubated for 20 hours with nicotine followed by incubation for 4 hours with ^{32}P i in the absence of nicotine; and [D], a single sample incubated in the presence of nicotine for 20 hours followed by incubation for 4 hours with ^{32}P i in the presence of nicotine. The relative phosphorylation of $\alpha 4_i$ was determined by normalizing the ^{32}P signals from the autoradiographs to the protein levels determined from the immunoblots. Data are expressed relative to controls to account for variations in immunoblot signals from different batches of antisera and the variable amount of protein expression in the cells. Values in the bar graph represent group means + S.E.M. from 9 experiments. *Significant difference from controls, $p < 0.05$; ‡, significant difference from group values for samples in which nicotine was present only during the labeling period, $p < 0.05$.

Fig. 4.

Effects of nicotine and protein kinase activation on the phosphorylation of $\alpha 4_i$ and $\alpha 4_m$ subunits. SH-EP1-h $\alpha 4\beta$ 2 cells were incubated in the absence (open bars) or presence (hatched bars) of 50 nM nicotine for 24 hours in the absence of $^{32}\text{P}_i$, washed, and incubated for 4 hours with $^{32}\text{P}_i$. Forskolin (10 μM in 0.1% DMSO) or PDBu (200 nM in 0.1% DMSO) were added during the last 15 or 30 minutes of incubation, respectively. The $\alpha 4$ subunits were immunoprecipitated from detergent-solubilized membranes and processed for autoradiography and immunoblotting as for Figure 3. A representative autoradiograph and immunoblot are shown. The relative phosphorylation of both $\alpha 4_i$ and $\alpha 4_m$ (^{32}P signal quantified from the autoradiograph/protein level determined from the immunoblot) was normalized to controls. Values in the bar graph represent group means + S.E.M. from 5–7 experiments. *Significant difference from corresponding controls, $p < 0.05$; †, significant difference from group values for samples incubated in the absence or presence of nicotine, $p < 0.05$.

Fig. 5.

Two-dimensional phosphopeptide maps and phosphoamino acid analyses of α_4 following incubation of cells with nicotine or PDBu. SH-EP1- $\alpha_4\beta$ 2 cells were labeled with ^{32}P i and incubated in the absence or presence of 50 nM nicotine or 200 nM PDBu for 30 minutes. Proteins were immunoprecipitated and separated as in Figure 3. The ^{32}P -labeled immature α_4 species bands were excised from the PVDF membranes and processed for either 2D-phosphopeptide mapping or phosphoamino acid analysis. For the former, samples were digested with trypsin, and samples containing equal amounts of radioactivity were spotted on TLC plates. Phosphopeptides were separated by electrophoresis in the horizontal dimension and ascending chromatography in the vertical dimension, and detected by autoradiography. Phosphopeptide signals grouped within clusters are depicted, representing previously identified phosphopeptide fragments generated from trypsinized α_4 subunit protein (Pollock et al., 2007;2009). For phosphoamino acid analysis, samples were digested with acid and resuspended in buffer containing the standards phosphoserine (S),

phosphothreonine (T), and phosphotyrosine (Y). Samples containing equal amounts of radioactivity were spotted onto TLC plates and were subjected to electrophoresis in the horizontal direction followed by the vertical dimension. The migration of the standards was visualized with ninhydrin. The phosphorylation of S, T, or Y was identified based on co-migration of the ^{32}P -labeled residues with stained phosphoamino acid standards.