Published in final edited form as:

Neuroscience. 2009 February 18; 158(4): 1311–1325. doi:10.1016/j.neuroscience.2008.11.032.

Cyclic AMP-dependent Protein Kinase A and Protein Kinase C Phosphorylate $\alpha 4\beta 2$ Nicotinic Receptor Subunits at Distinct Stages of Receptor Formation and Maturation

Veronica V. Pollock¹, Tina Pastoor¹, Christopher Katnik², Javier Cuevas², and Lynn Wecker^{1,2}

¹ Department of Psychiatry and Behavioral Medicine, University of South Florida College of Medicine, Tampa, Florida 33612-4799

² Department of Molecular Pharmacology and Physiology, University of South Florida College of Medicine, Tampa, Florida 33612-4799

Abstract

Neuronal nicotinic receptor $\alpha 4$ subunits associated with nicotinic $\alpha 4\beta 2$ receptors are phosphorylated by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), but the stages of receptor formation during which phosphorylation occurs and the functional consequences of kinase activation are unknown. SH-EP1 cells transfected with DNAs coding for human $\alpha 4$ and/or $\beta 2$ subunits were incubated with ³²Pi, and PKA or PKC were activated by forskolin or phorbol 12,13-dibutyrate, respectively. Immunoprecipitation and immunoblotting of proteins from cells expressing $\alpha 4\beta 2$ receptors or only $\alpha 4$ subunits were used to identify free $\alpha 4$ subunits, and $\alpha 4$ subunits present in immature $\alpha 4\beta 2$ complexes and mature $\alpha 4\beta 2$ pentamers containing complex carbohydrates. In the absence of kinase activation, phosphorylation of $\alpha 4$ subunits associated with mature pentamers was 3 times higher than subunits associated with immature complexes. PKA and PKC activation increased phosphorylation of free $\alpha 4$ subunits on different serine residues; only PKC activation phosphorylated subunits associated with mature $\alpha 4\beta 2$ receptors. Activation of both PKA and PKC increased the density of membrane-associated receptors, but only PKC activation increased peak membrane currents. PKA and PKC activation also phosphorylated $\beta 2$ subunits associated with mature $\alpha 4\beta 2$ receptors. Results indicate that activation of PKA and PKC leads to the phosphorylation $\alpha 4\beta 2$ receptors at different stages of receptor formation and maturation and has differential effects on the expression and function of $\alpha 4\beta 2$ receptors.

Keywords

protein kinase A; protein kinase C; neuronal nicotinic receptor; phosphorylation; receptor formation; receptor function

Neuronal nicotinic acetylcholine (ACh) receptors composed of $\alpha 4$ and $\beta 2$ subunits are widely distributed throughout the brain and represent the most abundant nicotinic receptor type in the

Address correspondence to: Lynn Wecker, Department of Psychiatry & Behavioral Medicine, USF College of Medicine, MDC Box 14, 3515 East Fletcher Avenue, Tampa, Florida 33613, Voice 813 974-5189, Fax 813 974-3236, email: lwecker@health.usf.edu.
Section Editor to whom manuscript should be sent for handling: Dr. Constantino Sotelo

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

brain (Zoli et al., 1995). These $\alpha 4\beta 2$ receptors participate in numerous biochemical and physiological processes, have been implicated in several neurological and behavioral disorders including nocturnal frontal lobe epilepsy and Alzheimer's disease, and may be responsible for the rewarding and addictive effects of nicotine (Picciotto et al., 2001; Tapper et al., 2004).

Studies have suggested that the expression and function of $\alpha 4\beta 2$ receptors are regulated post-translationally through phosphorylation/dephosphorylation mechanisms involving both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) (Rothhut et al., 1996; Eilers et al., 1997; Gopalakrishnan et al., 1997; Fenster et al., 1999; Jeanclos et al., 2001; Nashmi et al., 2003; Exley et al., 2006). Initial studies using M10 fibroblasts stably transfected with chicken $\alpha 4$ and $\beta 2$ subunit cDNAs indicated that activation of PKA by forskolin increased the number of cell surface receptors 2-fold (Rothhut et al., 1996). Furthermore, recent findings using rat $\alpha 4\beta 2$ receptors expressed in tsA201 cells have suggested that this increase may be attributed to the PKA-dependent phosphorylation of $\alpha 4$ subunits, which promotes association with the 14-3-3 chaperone protein, increasing steady-state levels of the subunit, and resulting in a 20% increase in cell surface receptor expression (Jeanclos et al., 2001). Similar studies using mouse $\alpha 4\beta 2$ receptors expressed in HEK293T cells have shown that activation of PKC also promotes the assembly and translocation of receptors to the cell surface (Nashmi et al., 2003) although the role of $\alpha 4$ subunit phosphorylation in mediating this action was not determined.

The phosphorylation of $\alpha 4$ subunits following activation of PKA and PKC has been demonstrated in several systems and evidence indicates that these kinases phosphorylate both unique and common sites located within the M3/M4 cytoplasmic domain of the subunit (Nakayama et al., 1993; Hsu et al., 1997; Wecker et al., 2001; Viseshakul et al., 1998; Guo and Wecker, 2002; Pacheco et al., 2003). Further, recent studies have suggested that these kinases may have a differential effect on the $\alpha 4$ subunit protein during different stages in the maturation of the $\alpha 4\beta 2$ pentamer. Pollock et al. (2007) have shown that two forms of $\alpha 4$ subunit protein can be immunoprecipitated from SH-EP1 cells transiently transfected with the DNAs coding for human $\alpha 4$ and $\beta 2$ subunits, a predominant 71–75 kDa protein and a less abundant protein at 80–85 kDa, the latter present most often in samples containing large amounts of protein. Further, activation of both PKA and PKC increased phosphorylation of the major 71–75 kDa protein, whereas phosphorylation of the higher molecular weight $\alpha 4$ protein was unaltered following activation of PKA, but increased following activation of PKC. Based on studies suggesting that the lower molecular weight $\alpha 4$ protein represents immature $\alpha 4$ subunit species while the higher molecular weight protein represents subunits isolated from mature $\alpha 4\beta 2$ pentamers (Whiting and Lindstrom 1988; Vallejo *et al.* 2005; Sallette *et al.* 2005), results suggest that PKA activation may increase phosphorylation of $\alpha 4$ subunits prior to formation of mature receptors, whereas PKC activation may have a dual action, phosphorylating $\alpha 4$ subunits both prior to after formation of the mature receptor. Thus, these studies investigated the phosphorylation of $\alpha 4$ subunits by PKA and PKC at distinct stage(s) in the assembly and maturation of $\alpha 4\beta 2$ receptors, and determined whether activation of PKA and PKC have different functional consequences.

EXPERIMENTAL PROCEDURES

Materials

The monoclonal antibodies used for immunoprecipitation included mAb299 (M218) raised against the extracellular loop of the rat nicotinic receptor $\alpha 4$ subunit and mAb290 (N8533) raised against the extracellular loop of the rat nicotinic receptor $\beta 2$ subunit, both of which cross react with the human subunits (Sigma-Aldrich Co.). The polyclonal antibodies used for immunoblotting were H-133 (sc-5591), a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 342–474 of the human $\alpha 4$ subunit, H-92

(sc-11372), a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 342–433 of the human $\beta 2$ subunit, and goat anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (sc-2030), purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sulfo-NHS-LC-Biotin was purchased from Pierce (Rockford, IL), endoglycosidase-H (Endo-H) and peptide N-glycanase F (PNGase F) were bought from New England Biolabs (Beverly, MA), and Lipofectamine™ 2000 and cell growth media were purchased from Invitrogen Corporation (Carlsbad, CA). Extravidin-HRP (E2886), Protein-G Sepharose and molecular markers were purchased from Sigma-Aldrich Co., and thin-layer chromatography (TLC) plates were obtained from VWR International Inc. (Bristol, CT). [^{32}P] and [^3H]cytosine were purchased from Perkin Elmer Life and Analytical Sciences Inc. (Boston, MA), polyvinylidene fluoride (PVDF) membranes (Immobilon-P) from Millipore Corporation (Bedford, MA), and XAR film from Eastman Kodak Co. (Rochester, NY). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Piscataway, NJ), basic electrophoresis chemicals from Bio-Rad Laboratories, Inc. (Hercules, CA), and sequencing grade trypsin from Roche Diagnostics Corp. (Indianapolis, IN). Human $\alpha 4$ and $\beta 2$ acetylcholine receptor subunit cDNA clones in pSP64 (kindly provided by Dr. Jon Lindstrom, Department of Neuroscience, University of Pennsylvania, Philadelphia, PA) were used to make constructs in pcDNA 3.1/hygro(–) and pcDNA 3.1 zeo(+), respectively (Pollock et al., 2007). SH-EP1 and SH-EP1- $\alpha 4\beta 2$ cells were kindly provided by Dr. Ron Lukas (Barrow Neurological Institute, Phoenix, AZ).

Transfection, ^{32}P i labeling, kinase activation, and preparation of whole cell lysates

SH-EP1 cells were grown in 60 mm dishes at 37°C in 5% CO_2 and maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM) supplemented with 5% fetal clone II, 10% horse serum, 1 mM sodium pyruvate, 8 mM L-glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 50 $\mu\text{g}/\text{ml}$ amphotericin B. Transfections were performed with cells at 90–95% confluency in Opti-MEM. Cells were incubated with 20 μl of Lipofectamine mixed with 8 μg of DNA for $\alpha 4$ only, $\beta 2$ only, or a mixture ($\alpha:\beta$, 2:3) for 2–3 hours at 37°C and for an additional 3–4 hours at 32°C. The Lipofectamine was removed and cells were incubated with growth media lacking antibiotics for an additional 18–20 hours at 32°C, for a total incubation time of 24 hours.

To label endogenous ATP stores, cells were incubated with ^{32}P i (1 or 2 mCi/ml) for 4 hours in phosphate-free DMEM. To produce maximal activation of PKA or PKC, 10 μM forskolin or 200 nM phorbol 12,13-dibutyrate (PDBu) in 0.1% DMSO were added during the final 15 or 30 minutes, respectively. These concentrations and times of incubation were chosen based on studies indicating that these parameters led to maximal activation of PKA by forskolin and PKC by PDBu without altering the amount of radioactivity taken up by cells during the labeling period. 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 500 μl 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 sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 U/ml aprotinin, and 2% Triton X-100 or 1% Lubrol. Samples were transferred into microfuge tubes, were triturated (20 strokes) using a 1 ml syringe with a 26-gauge needle, were further solubilized for 30 minutes at 4°C on a rotator, and were centrifuged at 100,000 $\times g$ for 10 minutes; the supernatants were withdrawn and transferred to tubes for further experimentation.

Immunoprecipitations

$\alpha 4$ subunit protein was immunoprecipitated from whole cell lysates using both the anti- $\alpha 4$ monoclonal antibody mAb299 and the anti- $\beta 2$ monoclonal antibody mAb290. Studies have shown that mAb299 immunoprecipitates subunits associated only with immature receptors

from transfected HEK293 cells expressing $\alpha 4\beta 2$ receptors, whereas mAb290 immunoprecipitates subunits associated with both immature and plasma membrane-associated mature receptors (Sallette et al., 2005). Whole cell lysates were pre-cleared with 15 μ l protein-G Sepharose for 30 minutes at 4°C. The samples were incubated with primary antibody (5 μ g of mAb299 or mAb290) overnight with rocking at 4°C, followed by rocking with 30 μ l protein-G sepharose for 3–4 hours. The protein/antibody/bead complexes were washed five times with 50 mM MOPS buffer (pH 7.2) containing 1 mM EDTA, 1 mM EGTA, and 0.2% Triton X-100, protein was eluted by boiling in Laemmli buffer (Laemmli, 1970), and samples were centrifuged at $2,000 \times g$ for 5 minutes to remove the protein G beads.

For sequential immunoprecipitations, samples were incubated with either mAb290 or mAb299 and protein-G sepharose as above, and centrifuged at $2,000 \times g$ for 5 minutes. The supernatants were reincubated with antibody and protein-G sepharose and the protein/antibody/bead complexes were processed as described. This procedure was repeated 2 more times for a total of 4 sequential immunoprecipitations.

To isolate $\alpha 4$ subunits that were not associated with $\beta 2$ subunits from cells expressing $\alpha 4\beta 2$ receptors, all $\alpha 4\beta 2$ complexes were immunoprecipitated from pre-cleared cell lysates by sequential incubation with mAb290 and excess (10 μ g) H-92, a polyclonal anti- $\beta 2$ antibody that is not conformation or assembly dependent. Samples were incubated with mAb290 and protein-G sepharose as above, centrifuged at $2,000 \times g$ for 5 minutes, and the supernatants incubated with H-92 and protein-G sepharose. Samples were centrifuged at $2,000 \times g$ for 5 minutes, and the supernatant from this second immunoprecipitation was mixed with additional protein-G sepharose to remove excess antibody, and centrifuged. The supernatants were incubated with mAb299 to immunoprecipitate $\alpha 4$ subunits, and samples processed as described.

Surface protein biotinylation and endoglycosidase cleavage

Twenty-four hours following transfections, cells were washed with ice cold PBS and incubated in 2 ml of ice cold PBS (pH 7.5) containing 0.5 mM Sulfo-NHS-LC-Biotin for 30 minutes at 4°C to biotinylate cell surface receptors. Reagent was removed by washing the cells with PBS containing 100 mM glycine, transferring them to microfuge tubes and washing twice more. Samples were centrifuged at $1,200 \times g$ for 5 minutes, the cells were resuspended in 500 μ l of homogenization/lysis buffer, and whole cell lysates prepared as described. Receptor complexes were immunoprecipitated from biotinylated and non-biotinylated lysates with mAb290 and protein eluted by boiling for 10 minutes with 65 μ l Laemmli buffer (Laemmli, 1970) containing 1% sodium dodecyl sulfate (SDS). The eluted samples were divided into three 20 μ l aliquots. One aliquot served as the control, and the others were subjected to endoglycosidase cleavage using both PNGase, which removes all N-linked carbohydrates, and Endo-H, which removes only high mannose and some hybrid types of N-linked carbohydrates. Thus, the second aliquot was mixed with 2 μ l of Endo-H, and the third was mixed with 2 μ l of PNGase F and 1 μ l of 10% NP-40 (nonyl phenoxypolyethoxyethanol), a nonionic, non-denaturing detergent that protects the PNGase F from inhibition by the SDS present. The samples were mixed thoroughly and incubated at 37°C for one hour, followed by the addition of 5 μ l of 20% SDS. Samples were frozen at -80°C until analysis by immunoblotting.

Autoradiography and immunoblotting

Immunoprecipitated proteins were separated on a 7.5% SDS-polyacrylamide gel (SDS-PAGE) and transferred to PVDF membranes. [^{32}P]-labeled $\alpha 4$ and $\beta 2$ subunit proteins were visualized by autoradiography by exposing the membranes to Kodak XAR film in the presence of intensifying screens at -80°C for 2–24 hours prior to immunoblotting.

For immunoblots, the PVDF membranes were blocked with Tris-buffered saline (TBS) containing 0.05% Tween-20 and 5% non-fat dry milk for 2 hours at 24°C. For samples in which both $\alpha 4$ and $\beta 2$ subunit proteins were determined, membranes were cut horizontally just above the 59 kDa marker between the $\alpha 4$ and $\beta 2$ protein bands. Membranes were incubated with H-133 and H-92 (each 200 $\mu\text{g}/\text{ml}$ at 1:2000 dilution) in TBS/0.05% Tween/0.5% non-fat dry milk for 1 hour at 24°C to immunodetect the $\alpha 4$ and $\beta 2$ subunit proteins, respectively. These polyclonal antibodies have been shown to react specifically with the proteins of interest following immunoprecipitation with the monoclonal antibodies and were used to avoid high backgrounds obtained when the monoclonal antibodies are used for both immunoprecipitation and immunodetection (Pacheco et al., 2003; Pollock et al., 2007). The membranes were washed and incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (400 $\mu\text{g}/\text{ml}$ at 1:2000 dilution) for 30 minutes, and the $\alpha 4$ and $\beta 2$ signals were visualized using ECL. Autoradiographs and immunoblots were quantitated by densitometry using a Bio-Rad Imaging Densitometer with Multi-Analyst software (Bio-Rad Laboratories). Relative phosphorylation was calculated by normalizing the densitometric signals from the autoradiographs to the immunoblots.

Phosphopeptide mapping

Two-dimensional (2D) phosphopeptide mapping of the immunoprecipitated [^{32}P]-labeled $\alpha 4$ subunits was performed using standard techniques (Boyle et al., 1991) as described in detail by Pollock et al. (2007). Briefly, PVDF membrane bands containing the [^{32}P]-labeled $\alpha 4$ protein were excised, 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 counts and not equal amounts of protein were analyzed.

Radioligand binding

SH-EP1- $\alpha 4\beta 2$ cells were maintained (in 60 mm dishes) at 37°C in 5% CO_2 and grown 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 $\mu\text{g}/\text{ml}$ streptomycin, 0.05 mg/ml amphotericin B, 0.5 mg/ml zeocin and 0.4 mg/ml hygromycin B. Cells were kept in continuous culture, fed every 2–3 days, and used at 85% confluency. Cells were incubated in control media or media containing 10 μM forskolin or 200 nM PDBu (dissolved in 0.1% DMSO) for 24 hours. Following incubation, cells were washed 3 times with PBS, and transferred to a Dounce homogenizer. Membrane fractions were prepared by homogenizing cells in 50 mM Tris-HCl buffer (pH 7.0) containing 120 mM NaCl, 5 mM KCl, 1 mM MgCl_2 and 2.5 mM CaCl_2 . Cell lysates were centrifuged at $500 \times g$ for 5 minutes at 4°C and the resultant supernatants were recentrifuged at $20,800 \times g$ for 30 minutes at 4°C; the pellets were washed 3 times and resuspended in Tris-HCl buffer. The binding reaction was initiated by the addition of membrane preparations (5–10 μg protein) to triplicate sets of tubes containing 2.5 nM [^3H]cytisine (32–35 Ci/mmol), representing $4 \times K_d$ (Pacheco et al., 2001) and Tris-HCl buffer. Tubes were incubated for 2 hours on ice and bound ligand separated from free ligand by rapid filtration over Whatman GF/B filters, which were pre-soaked in Tris-HCl to minimize non-specific binding. Tubes and filters were rinsed twice with ice-cold Tris-HCl buffer, filters placed in scintillation vials and 8 ml of scintillation fluid added to each vial. Samples were shaken mechanically for 1 hour and radioactivity was determined in a Beckman LS-6000 scintillation counter (Beckman Counter, Fullerton, CA). Non-specific binding was measured in the presence of 10 μM nicotine and specific binding calculated as the difference between total and non-specific binding. Experiments were performed using different passages of cultured cells.

Electrophysiology

SH-EP1- $\alpha 4\beta 2$ cells were plated on glass cover slips and maintained in DMEM (high glucose) as above. Cover slips were transferred to a recording chamber (0.5 ml) mounted on an inverted phase-contrast microscope that allowed isolated cells to be identified. Membrane currents were studied under voltage-clamp mode using the amphotericin B perforated-patch variation of the whole-cell patch-clamp technique. Patch electrodes were pulled from thin-walled borosilicate glass (World Precision Instruments Inc., Sarasota, FL) using a Sutter Instrument (Novato, CA) P-87 pipette puller and had resistances of 1–1.5 M Ω . Membrane currents were amplified and filtered (5 kHz) using an Axopatch 200B patch-clamp amplifier, digitized with a Digidata 1322 B, and acquired using Clampex 8.2 (Axon Instruments, Foster City, CA). The external solution for whole-cell recordings was physiological saline solution containing 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 1.6 mM MgCl₂, 7.7 mM glucose and 10 mM HEPES (pH 7.2 with 1 M NaOH). The intracellular solution contained 75 mM CsSO₄, 55 mM CsCl, 5 mM MgSO₄, 10 mM HEPES, 360 mg/ml amphotericin B, and 0.6% DMSO (pH 7.2 with *N*-methyl-D-glucamine). ACh was applied via a gravity-fed rapid application system. The flow of solution was controlled by solenoid valves (General Valve, Fairfield, NJ) using a Master 8 channel stimulator (A.M.P.I., Jerusalem, Israel) and was directed by a piezoelectric bimorph connected to a voltage generator (Burleigh Instruments, Inc., Fishers, NY). The rate of solution change was determined to be less than 5 msec as measured by liquid junction potential changes. Increasing concentrations of ACh were added every 4 minutes, a time interval determined to be sufficient for recovery from the desensitization induced by prior exposure to agonist.

Data Analysis

Where appropriate, data are presented as mean $\pm$ s.e.m. The Student's *t*-test was used for comparisons involving two groups. ANOVA was used for more than 2 groups, and 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.

For electrophysiological experiments, data were analyzed using SigmaPlot 8 and SigmaStat 3.1 (Systat Software Inc., Point Richmond, CA). Data were normalized to peak responses obtained for each cell at 3 mM ACh, and concentration-response data were fit using the single- $[y = (E_{\max})([x]^n)/(EC_{50}^n + [x]^n)]$ or two-site $[y = (E_{\max 1})([x]^{n1})/(EC_{501}^{n1} + [x]^{n1}) + (E_{\max 2})([x]^{n2})/(EC_{502}^{n2} + [x]^{n2})]$ Langmuir-Hill Equations, where $E_{\max}$ represents the maximal response, $[x]$ is the agonist concentration, EC_{50} is the apparent macroscopic equilibrium constant, and n is the Hill Coefficient.

RESULTS

Identification and cellular localization of $\alpha 4$ and $\beta 2$ subunits at different stages of receptor assembly and maturation

Initial experiments established and validated conditions to separate and identify $\alpha 4$ and $\beta 2$ subunits at distinct stages in the assembly and maturation of $\alpha 4\beta 2$ receptors. The first experiment compared the ability of mAb299 and mAb290 to immunoprecipitate subunits from whole cell lysates prepared from SH-EP1 cells transfected with $\alpha 4$ and $\beta 2$ subunit DNAs either separately or in combination. Results (Fig. 1[A]) demonstrate that when samples were immunoprecipitated with mAb299 and probed for $\alpha 4$, a predominant signal corresponding to an apparent molecular weight of 70–75 kDa and a minor signal at approximately 66 kDa (observed only when samples contained large amounts of protein) were apparent for samples from cells expressing either $\alpha 4$ subunits only (lane 1) or both $\alpha 4$ and $\beta 2$ subunits (lane 2). In contrast, when samples were immunoprecipitated with mAb290, cells expressing both subunits (lane 4) exhibited both the 70–75 and 66 kDa $\alpha 4$ protein bands, as well as a higher molecular

weight signal at 80–85 kDa. No $\alpha 4$ signals were apparent in samples from cells expressing only $\beta 2$ subunits (lane 3). When samples immunoprecipitated with mAb299 were probed for $\beta 2$, a single signal was apparent at 47 kDa in cells expressing both subunits (lane 2); no signal was present in cells expressing only the $\alpha 4$ subunit (lane 1). When mAb290 was used for immunoprecipitation, samples from cells expressing both subunits (lane 4) exhibited both the 47 kDa $\beta 2$ protein and a higher molecular weight signal at 50 kDa. Samples from cells expressing only $\beta 2$ subunits (lane 3) yielded a very faint signal at 47 kDa. No signals were present for either $\alpha 4$ or $\beta 2$ in samples from untransfected cells (data not shown), confirming the specificity of the antibodies used.

It is possible that the higher molecular weight signals for both $\alpha 4$ and $\beta 2$ subunits present in samples immunoprecipitated with mAb290 (80–85 kDa for $\alpha 4$ and 50 kDa for $\beta 2$) correspond to subunits isolated from mature $\alpha 4\beta 2$ receptors with fully processed sugars, while the lower molecular weight signals (70–75 kDa for $\alpha 4$ and 47 kDa for $\beta 2$) arise from immature $\alpha 4\beta 2$ complexes containing high mannose (Sallette et al., 2005). The minor third $\alpha 4$ band of 66 kDa likely corresponds to a fully deglycosylated degradation product (Sallette et al., 2005). To confirm the identity of these signals, lysates from cells expressing both subunits were prepared, and $\alpha 4\beta 2$ complexes were either immunoprecipitated immediately with mAb290 followed by digestion with Endo-H or PNGase F to remove predominantly high mannose carbohydrates or all N-linked carbohydrates, respectively, or were surface biotinylated with Sulfo-NHS-LC-biotin prior to immunoprecipitation and glycosidase digestion, and probed with extravidin-conjugated peroxidase to recognize only biotinylated samples (Fig. 1[B]). Immunoblots of non-biotinylated samples indicate that the higher molecular weight species of both $\alpha 4$ and $\beta 2$ were partially Endo-H resistant, but completely digested by PNGase F, indicating that the proteins represented by these bands contain 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 ($\alpha 4_m$ and $\beta 2_m$). This was confirmed by the immunoblots for the biotinylated samples indicating that extravidin-conjugated peroxidase recognized only the higher molecular weight protein bands, corresponding to subunits isolated from cell surface, fully-processed mature receptors, in agreement with Vallejo et al. (2005). The lower molecular weight species of the subunits, which were present only in the non-biotinylated samples, exhibited identical digestion profiles for Endo-H and PNGase F, indicating that the 70–75 kDa $\alpha 4$ protein and 47 kDa $\beta 2$ protein contain only high mannose corresponding to subunits present in immature complexes located primarily in the endoplasmic reticulum ($\alpha 4_i$ and $\beta 2_i$). Results further demonstrate that following PNGase F cleavage, $\alpha 4_i$ migrates at an apparent molecular weight of about 66 kDa, supporting the idea that the lowest molecular weight $\alpha 4$ protein signal in Fig. 1[A] corresponds to a deglycosylated form of the subunit.

To ascertain whether mAb290 immunoprecipitated $\alpha 4$ subunits from all immature $\alpha 4\beta 2$ complexes, and determine whether solubilization in buffer containing Triton X-100 could have affected the subunit species detected (Kuryatov et al., 2005), sequential immunoprecipitations were performed in cell lysates prepared using either Triton X-100 or Lubrol, the latter less protein complex dissociating (Schuck et al., 2003). Results (Fig. 1[C] left immunoblot) indicate that a single immunoprecipitation with mAb290 from Triton-X solubilized samples complexed all $\alpha 4_m$, but a second immunoprecipitation with mAb290 was necessary to isolate what appeared to be the remainder of the 70–75 kDa $\alpha 4$ species because no signal was detected upon a third immunoprecipitation. However, when the supernatant remaining after the third immunoprecipitation with mAb290 was incubated with mAb299, a prominent signal was evident at 70–75 kDa. Because mAb290 immunoprecipitates only $\alpha 4$ subunits coupled to $\beta 2$ subunits, results suggest that the 70–75 kDa $\alpha 4$ band following mAb299 immunoprecipitation may represent both free $\alpha 4$ subunits and subunits present in immature $\alpha 4\beta 2$ complexes in a conformation unable to bind mAb290. Thus, although mAb290 complexes all $\alpha 4$ protein

present in mature $\alpha 4\beta 2$ receptors upon a single immunoprecipitation, it may not bind to $\alpha 4$ protein in all immature $\alpha 4\beta 2$ complexes. The idea that immature $\alpha 4\beta 2$ complexes exist in at least 2 conformations, only one of which can be complexed by mAb290 and may represent a late developmental complex, with the other bound by mAb299 representing a more juvenile subunit complex (Sallette et al., 2005), is supported by results indicating that the 47 kDa $\beta 2$ signal was still apparent in samples immunoprecipitated by mAb290 three times followed by immunoprecipitation with mAb299. These findings were verified further by similar experiments in which Triton-X solubilized samples were immunoprecipitated sequentially with mAb299 three times prior to mAb290 (Fig. 1[C] right immunoblot). Lastly, when samples prepared in Triton X-100 were compared with those in Lubrol (Fig. 1[C] center immunoblot), results indicate that Triton X-100 did not affect the subunit species detected as results with Triton X-100 were identical to those obtained using Lubrol.

$\alpha 4$ subunits associated with immature $\alpha 4\beta 2$ complexes are phosphorylated by both PKA and PKC, whereas subunits associated with mature pentameric receptors are phosphorylated only by PKC

To investigate the possibility that activation of PKA and PKC may increase $\alpha 4$ subunit phosphorylation at different stages of receptor assembly and maturation, SH-EP1 cells were transfected with $\alpha 4$ and $\beta 2$ subunit DNAs and incubated with ^{32}P i for 4 hours, the final 15 or 30 minutes in 10 μM forskolin or 200 nM PDBu, respectively. Whole cell lysates were incubated with mAb290 to immunoprecipitate $\alpha 4\beta 2$ complexes, proteins were separated by electrophoresis and transferred to PVDF membranes, and the immunoblots probed for $\alpha 4$ and $\beta 2$ subunit protein (Fig. 2). In the absence of exogenous stimulation of the kinases (basal state), both $\alpha 4_m$ and $\alpha 4_i$ were phosphorylated, the former to a greater extent (3 times) than the latter on a protein basis. Stimulation of PKA by forskolin did not alter the relative phosphorylation of $\alpha 4_m$, but significantly ($p < 0.05$) increased $\alpha 4_i$ phosphorylation 2.2-fold. In contrast, stimulation of PKC by PDBu increased phosphorylation of both $\alpha 4_m$ and $\alpha 4_i$ significantly ($p < 0.05$) 1.6-fold for both species. Furthermore, when the possible phosphorylation of $\beta 2$ subunits was investigated, results indicated that only subunits isolated from mature receptors were phosphorylated, and stimulation of either PKA or PKC increased phosphorylation of $\beta 2_m$ significantly ($p < 0.05$) by approximately 3-fold; immature $\beta 2$ subunits were not phosphorylated under any condition (see autoradiograph and immunoblot in Fig. 2). Results demonstrate for the first time that $\beta 2$ subunits associated with mature $\alpha 4\beta 2$ receptors are also subject to phosphorylation upon kinase activation, albeit the relative phosphorylation of $\beta 2_m$ in the basal state was only 1/10th that of $\alpha 4_m$.

To determine whether PKA and PKC phosphorylated the same or similar residues on $\alpha 4$ subunits associated with mature receptors and immature complexes, the [^{32}P]-labeled bands were excised from the PVDF membranes, digested with trypsin, and equal amounts of radioactivity (500 cpm) per sample were spotted on TLC plates and 2D phosphopeptide maps generated. Results (Fig. 3) demonstrate that under basal conditions, 4 clusters (C1–C4) of labeled phosphopeptide fragments were present in $\alpha 4_m$, with a predominant signal at C1. This signal was also apparent for $\alpha 4_i$, but the other signals were faint because basal phosphorylation for $\alpha 4_i$ was only 1/3 that of $\alpha 4_m$ (Fig. 2). When subunit species were isolated from samples incubated with forskolin, the phosphopeptide pattern generated for $\alpha 4_m$ did not differ from controls, whereas the map generated for $\alpha 4_i$ had increased signals associated with both C1 and C4, indicating that the substrates contained within these clusters are phosphorylated upon PKA activation prior to receptor maturation. When $\alpha 4$ subunit species were isolated from samples following incubation with PDBu, the map for $\alpha 4_m$ exhibited increased the phosphorylation of fragments within C3 and C4, whereas the pattern for $\alpha 4_i$ demonstrated increased phosphorylation associated with C3 and the appearance two additional phosphopeptide fragments, C5 and C6, which were not present in $\alpha 4_m$, suggesting that these latter sites are

phosphorylated upon activation of PKC prior to receptor maturation, and are dephosphorylated during the maturation process.

PKA and PKC can phosphorylate $\alpha 4$ subunits prior to assembly with $\beta 2$ subunits

Results from sequential immunoprecipitation studies suggested that the 70–75 kDa $\alpha 4$ protein from samples immunoprecipitated with mAb299 likely represents both free $\alpha 4$ subunits (either monomers or oligomers) and subunits associated with early developmental immature $\alpha 4\beta 2$ complexes that can not be complexed by mAb290. To determine directly whether free $\alpha 4$ subunits can be phosphorylated either as monomers or oligomers, $\alpha 4$ subunit phosphorylation was determined in cells expressing only these subunits. SH-EP1 cells transfected with the DNA coding for $\alpha 4$ subunits only were incubated with ^{32}Pi for 4 hours, the final 15 or 30 minutes in the absence or presence of forskolin or PDBu, respectively, whole cell lysates were prepared, and $\alpha 4$ subunit protein was immunoprecipitated with mAb299. Autoradiographs and immunoblots (Fig. 4[A]) indicate that when $\alpha 4$ subunits are expressed alone, a single phosphorylated protein band of approximately 70–75 kDa is apparent, and PKA or PKC stimulation increases subunit phosphorylation significantly ($p < 0.05$) by 4.9- or 2.7-fold, respectively. The 2D phosphopeptide maps generated from these samples indicate that under basal conditions, phosphorylation is readily apparent on fragments within C1 and C4, and forskolin incubation increased phosphorylation of both of these substrates. In contrast, incubation with PDBu increased signals associated with fragments within C2, C3, C5 and C6, the latter particularly prominent. PKC stimulation did not alter the signals associated with either C1 or C4. Thus, the phosphopeptide maps for samples immunoprecipitated by mAb299 from cells expressing only $\alpha 4$ subunits were identical to those generated for $\alpha 4_i$ for samples immunoprecipitated by mAb290 from cells expressing both $\alpha 4$ and $\beta 2$ subunits under basal or PKA stimulated conditions, but not following activation of PKC.

Although results suggested that $\alpha 4$ subunits can be phosphorylated prior to assembly with $\beta 2$ subunits, $\alpha 4$ complexes never reach the cell surface (Ren et al., 2005) and $\alpha 4$ subunit expression in brain depends on $\beta 2$ subunit expression (Whiteaker et al., 2006). Thus, to ascertain whether $\alpha 4$ subunit phosphorylation in cells expressing only $\alpha 4$ subunits was the same as in cells expressing both subunits, following preparation of cell lysates from cells transfected with the cDNAs for both $\alpha 4$ and $\beta 2$ subunits and incubated as above, samples were incubated sequentially with mAb290 and excess of the anti- $\beta 2$ polyclonal antibody H-92 to complex all species associated with $\beta 2$ subunits. Immunoblot analysis confirmed that this procedure removed all $\alpha 4\beta 2$ complexes irrespective of conformation or assembly. The free $\alpha 4$ subunits remaining in the samples were immunoprecipitated with mAb299. Autoradiographs and immunoblots (Fig. 4[B]) demonstrate that free $\alpha 4$ subunits unassociated with $\beta 2$ subunits have the same molecular weight as the $\alpha 4$ protein isolated from cells expressing $\alpha 4$ subunits alone, albeit a much lower yield was obtained. Phosphorylation was apparent under basal conditions, and stimulation of PKA or PKC increased phosphorylation significantly ($p < 0.05$) by 1.9- or 1.7-fold, respectively, similar to the respective 2.2- or 1.6-fold increases observed for $\alpha 4_i$ following stimulation of PKA or PKC (Fig. 2). Due to the low protein yield obtained from these samples, corresponding 2D phosphopeptide maps could not be generated. However, based on the similarity of the relative increase in phosphorylation of free $\alpha 4$ subunits and $\alpha 4_i$ following PKA and PKC stimulation, data supports the idea that $\alpha 4$ subunits may be phosphorylated prior to assembly with $\beta 2$ subunits under basal and kinase-stimulated conditions.

Both PKA and PKC activation increase the number of membrane-associated receptors, but only PKC stimulation increases receptor function

Results indicating that both PKA and PKC phosphorylate $\alpha 4$ subunits prior to associating with $\beta 2$ subunits, whereas only PKC phosphorylates subunits associated with fully processed mature

receptors suggest that activation of these kinases may differentially affect receptor processing and function. To examine this possibility, both the number of membrane-associated receptors and magnitude of ACh-activated currents were determined in SH-EP1 cells stably transfected with human $\alpha 4$ and $\beta 2$ subunits (SH-EP1- $h\alpha 4\beta 2$ cells) and incubated with forskolin or PDBu for 24 hours. The first series of experiments determined the effects of forskolin and PDBu on receptor density. Based on studies indicating that SH-EP1- $h\alpha 4\beta 2$ cells express a single population of binding sites with high affinity and 24 hours incubation with nicotine increases receptor density (Pacheco et al., 2001), the binding of 2.5 nM [3 H]cytisine to membranes prepared from cells incubated in the absence or presence of 10 μ M forskolin or 200 nM PDBu for 24 hours was measured. Results (Fig. 5[A]) indicate that both forskolin and PDBu increased the number of membrane-associated receptors significantly ($p < 0.05$) by 1.6- and 1.4-fold, respectively, in agreement with studies from HEK293 cells stably expressing human $\alpha 4\beta 2$ receptors (Gopalakrishnan et al., 1997). Studies investigating shorter incubation times (6 and 12 hours) with forskolin and PDBu did not detect any alterations in receptor density (data not shown).

To evaluate the consequences of kinase activation on functional responses, ACh-evoked currents were measured in these cells, which have been shown to express functional receptors with properties similar to human $\alpha 4\beta 2$ receptors studied in other expression systems (Wu et al., 2004; Eaton et al., 2003). Initial experiments were conducted using the perforated-patch configuration of the whole-cell recording technique to determine the basic functional properties of the receptors expressed by these cells under conditions which preserve the intracellular milieu required for 'normal' kinase and phosphatase functioning. Application of ACh evoked fast activating transient inward currents that exhibited concentration-dependent amplitude and concentration- and time-dependent desensitization (Fig. 5[B]). When the normalized peak amplitude was plotted as a function of ACh concentration, a sigmoidal concentration-response relationship was apparent with data best fit by the two-site Langmuir-Hill equation with the following parameters: the high affinity site had $E_{\max-1}$, EC_{50-1} , and n_1 values of 0.11, 1 μ M, and 0.7, respectively; the low affinity site had $E_{\max-2}$, EC_{50-2} , and n_2 values of 0.89, 161 μ M, and 1.0, respectively. The voltage dependence of responses to 2 mM ACh were consistent with nicotinic receptor activation. A plot of peak ACh-evoked current amplitude as a function of holding potential demonstrated a linear current-voltage relationship at negative potentials, with a reversal potential near 0 mV, and inward rectification at positive potentials (Fig. 5[C]). When cells were incubated with 10 μ M forskolin or 200 nM PDBu for 24 hours, forskolin incubation did not alter peak ACh-evoked current densities, whereas incubation with PDBu increased current density significantly ($p < 0.05$) by nearly 2-fold (Fig. 5[D]).

To ascertain whether the ability of PDBu to increase ACh-evoked responses was attributed specifically to PKC activation, cells were incubated with PDBu in the absence or presence of the PKC inhibitor Ro-31-8220 (100 nM) for 24 hours. Results (Fig. 6[A]) indicated that Ro-31-8220 prevented the PDBu-induced increase. Incubation of cells with Ro-31-8220 alone or vehicle (0.1% DMSO) did not alter affect peak current amplitudes. Thus, the ability of PDBu to functionally up regulate $\alpha 4\beta 2$ receptors could be attributed specifically to activation of PKC.

Based on studies indicating that PKC activation for 30 minutes increases the proportion of mature $\alpha 4\beta 2$ receptors on or near the surface of HEK293T cells (Nashmi et al., 2003), the time dependence of the ability of PDBu to increase ACh-activated currents was investigated. SH-EP1- $h\alpha 4\beta 2$ cells were incubated with PDBu for 1 hour and peak ACh-evoked current densities were measured either immediately following a 10 minute washout or 23 hours following washout and incubation in control media. When cells were exposed to PDBu for 1 hour and responses measured following a 10 minute washout, no differences were apparent between control and PDBu exposed cells (Fig. 6[B]). In contrast, when cells were incubated with PDBu for 1 hour followed by a 23 hour incubation period in control media, a significant ($p < 0.05$)

55% increase in ACh-evoked current density was observed, and this increase was prevented by incubating cells with Ro-31-8220 (Fig. 6[C]). These results indicate that the ability of PDBu to increase receptor function appears to be initiated by activation of PKC, but requires additional cellular processes before functional differences are manifest.

DISCUSSION

The goals of this study were to determine whether activation of PKA and PKC increases the phosphorylation of $\alpha 4$ subunits during different stages in the assembly and maturation of $\alpha 4\beta 2$ receptors, and differentially affects receptor function. Results indicate that: 1) PKA phosphorylates $\alpha 4$ subunits on peptide fragments contained within C1 and C4 prior to complexing with $\beta 2$ subunits, and these sites remain phosphorylated during the development of immature $\alpha 4\beta 2$ complexes; 2) PKC phosphorylates $\alpha 4$ subunits on two substrates within C5 and C6 prior to complexing with $\beta 2$ subunits, and these sites remain phosphorylated during the development of immature $\alpha 4\beta 2$ complexes; 3) PKC also phosphorylates a fragment within C3 on $\alpha 4$ subunits associated with both immature $\alpha 4\beta 2$ complexes and mature receptors; and 4) activation of both kinases increases the number of membrane-associated receptors, but only activation of PKC increases ACh-evoked current density. Studies also demonstrate for the first time that $\beta 2$ subunits are phosphorylated, but only in the mature $\alpha 4\beta 2$ receptor in the plasma membrane.

Experiments to separate $\alpha 4$ species at distinct stages of receptor assembly and maturation led to several important observations, some, but not all of which are consistent with other studies. The finding that additional higher molecular weight signals for both $\alpha 4$ and $\beta 2$ subunits were present in samples immunoprecipitated with mAb290 and not mAb299 is identical to results from metabolic labeling studies indicating that the high molecular weight forms correspond to subunits isolated from mature $\alpha 4\beta 2$ receptors with fully processed sugars located on the plasma membrane, while the lower molecular weight signals (70–75 kDa for $\alpha 4$ and 47 kDa for $\beta 2$) arise from immature $\alpha 4\beta 2$ complexes containing high mannose (Sallette et al., 2005). In addition, results from the present study also confirm that mAb299 complexes $\alpha 4$ subunits expressed either alone or with $\beta 2$ subunits (Sallette et al., 2005), and extend this finding to indicate that this antibody immunoprecipitates two species of $\alpha 4$ present in both conditions, both the predominant 70–75 kDa immature species, and a lower molecular weight species of 66 kDa, likely representing a non-glycosylated form of $\alpha 4$ (Fig. 1[A]). However, in contrast to Sallette et al. (2005), who reported that mAb290 recognizes $\beta 2$ subunits only when co-expressed with $\alpha 4$ subunits, results from the present study indicate that mAb290 does not require $\beta 2$ to be in a heterooligomeric form with $\alpha 4$, as a signal for $\beta 2$, albeit faint, is present at 47 kDa in cells transfected with only the cDNA for $\beta 2$ subunit protein. It is possible that this difference reflects differences in the cell systems used, the methods of transfection, the amount of cDNA transfected, the time experiments were performed following transfection, or the relative expression level of the subunit. Nevertheless, because the signal for the $\beta 2$ subunit from cells transfected only with this subunit was so faint, results suggest that perhaps in this situation, heteromers of $\beta 2$ are formed that sterically hinder the ability of mAb299 to bind to the extracellular loop of the subunit.

Results from the current experiments also support findings that the anti- $\alpha 4$ monoclonal antibody mAb299 obtained from Sigma-Aldrich Co. (St. Louis, MO) does not immunoprecipitate subunits associated with mature $\alpha 4\beta 2$ receptors (Sallette et al., 2005). This observation was somewhat surprising as we have demonstrated that a different commercial preparation of mAb299 from Covance Inc. (Richmond, CA) did indeed complex the mature 80–85 kDa $\alpha 4$ species (Pollock et al., 2007). These antibodies were generated from the same clone, the only difference being that the preparation from Covance Inc. is generated and used in ascites fluid, whereas the preparation from Sigma-Aldrich Co. was generated in vitro and

contains purified antibody. Although one may expect the former to contain contaminants that bind to cellular membranes thereby preventing immunodetection, this was not the case, and the reason why these preparations differ in their ability to complex $\alpha 4$ subunits associated with mature receptors remains unclear.

Prior studies have identified two major substrate sites for PKA on $\alpha 4$ subunits, Ser467 on a peptide fragment within C1, and Ser362 on a fragment within the C4 doublet (Pollock et al., 2007). Based on the phosphopeptide patterns generated by Pollock et al. (2007), the present study indicates that Ser467 is phosphorylated on $\alpha 4$ subunits prior to complexing with $\beta 2$ subunits and remains phosphorylated throughout receptor assembly, maturation, and trafficking to the plasma membrane, suggesting that its phosphorylation plays a long-lasting determinant role. This idea is supported by studies demonstrating that forskolin stimulation of PKA increases the binding of the 14-3-3 chaperone protein to the rat $\alpha 4$ subunit expressed in tsA 201 cells, resulting in an increase in the steady-state level of $\alpha 4$ protein and the number of $\alpha 4\beta 2$ cell surface receptors, and when Ser441, which corresponds to Ser467 in the human $\alpha 4$ subunit, was mutated to an alanine, this effect was not present (Jeanclos et al., 2001). Furthermore, studies in *Xenopus* oocytes have shown that when Ser467 in human $\alpha 4$ subunits was replaced by alanine and expressed with $\beta 2$ subunits, steady-state concentrations of $\alpha 4$ subunit protein, the maximal density of [^3H]cytisine binding sites, ACh response amplitude, and the proportion of $\alpha 4\beta 2$ receptors with low sensitivity to agonist all decreased, and opposite effects were noted when Ser467 was replaced by aspartate to mimic the phosphorylated $\alpha 4$ subunit (Exley et al., 2006). Based on these findings, evidence suggests that phosphorylation of Ser467 on free $\alpha 4$ subunits prior to association with $\beta 2$ subunits has long-lasting consequences increasing the stability of $\alpha 4$ subunits, perhaps resulting in an enhanced expression of $\alpha 4\beta 2$ receptors in the low affinity $(\alpha 4)_3(\beta 2)_2$ configuration (Nelson et al., 2003; Moroni et al., 2006).

Similarly, based on prior phosphopeptide patterns (Pollock et al., 2007), the current results indicate that Ser362 also appears to be phosphorylated by PKA prior to assembly of $\alpha 4$ with $\beta 2$ subunits, but this residue is minimally phosphorylated on $\alpha 4$ subunits associated with mature receptors. This suggests that this post-translational modification plays a transient role prior to $\alpha 4\beta 2$ complexes exiting the endoplasmic reticulum. Although a functional role for the PKA-mediated phosphorylation of Ser362 has not been investigated, studies have identified sequences within the large M3/M4 cytoplasmic loop of $\alpha 4$ subunits that are overlapping with or adjacent to Ser362 that are essential for $\alpha 4\beta 2$ receptor subcellular targeting (Xu et al., 2006), export from the endoplasmic reticulum (Ren et al., 2005) or trafficking from the endoplasmic reticulum to the cell surface (Keller et al., 2001). It is possible that phosphorylation of Ser362 by PKA could play a role in regulating one or more of these functions. Although PKA is localized in the cytosol and is highly concentrated around the endoplasmic reticulum and Golgi (Nigam and Blobel, 1989), it does not appear to be available to phosphorylate any additional PKA sites on $\alpha 4$ subunits associated with immature complexes or fully mature receptors in the plasma membrane.

Possible substrate sites for PKC on $\alpha 4$ subunits include Ser550, which is present on one fragment contained within the C3 triplet (Pollock et al., 2007). Stimulation of PKC by PDBu enhanced the phosphorylation of this fragment within C3 on $\alpha 4$ subunits associated with both immature $\alpha 4\beta 2$ complexes and mature receptors (Fig. 3), but not on free $\alpha 4$ subunits (Fig. 4). This suggests that phosphorylation of Ser550 may affect both the trafficking of receptors from the endoplasmic reticulum to the cell surface and receptor function rather than the expression of a specific receptor conformation. The phosphorylation of $\alpha 4$ subunits within mature receptors at the plasma membrane by PKC is not surprising as phorbol esters induce translocation of the enzyme to the plasma membrane (Oancea and Meyer, 1998; Almholt et al., 1999; Shirai and Saito, 2002) or other cellular membranes (Wang et al., 2000), where it

can access and phosphorylate sites in the cytoplasmic domain of $\alpha 4$ subunits. Ser550 is unlike many other phosphorylation sites within the M3/M4 cytoplasmic domain of $\alpha 4$ subunits, which demonstrate a high degree of sequence homology among different species, and is present only on subunits from human and chicken, and not rat or mouse (see Fig. 1 in Pollock et al., 2007). Thus, the phosphorylation of Ser550 may be responsible for functional differences observed between human and rat receptors such as the human receptor exhibiting sensitization following sustained agonist exposure (Buisson and Bertrand, 2001; Gopalakrishnan et al., 1997) whereas the rat receptor exhibits inactivation (Fenster et al., 1999; Hsu et al., 1997).

In addition to the PKC-mediated phosphorylation of Ser550 on immature complexes and mature receptors, stimulation of this kinase led to the appearance of two additional unidentified phosphopeptide fragments within C5 and C6 on free $\alpha 4$ subunits (Fig. 4), and these sites remain phosphorylated on subunits associated with immature $\alpha 4\beta 2$ complexes, but not on those associated with mature receptors (Fig. 3). This pattern indicates that these residues are phosphorylated on $\alpha 4$ subunits in the endoplasmic reticulum prior to assembly with $\beta 2$ subunits, that phosphorylation persists throughout $\alpha 4\beta 2$ complex formation, but that the residues are dephosphorylated by the time the mature receptor is transported from the Golgi to the plasma membrane. Based on evidence that Ser550 is phosphorylated on subunits associated with immature complexes, in concert with findings that phosphorylation of Ser550 is greater on $\alpha 4$ subunits associated with mature pentamers, it is possible that dephosphorylation of the substrates on fragments within C5 and C6 must occur prior to further phosphorylation of Ser550. Thus, the phosphorylation and dephosphorylation of substrates within C5 and C6 is dependent on the state of assembly and maturation of the receptor with dephosphorylation likely involved in trafficking of receptors from the endoplasmic reticulum to the cell surface (Keller et al., 2001). A schematic summarizing the sites of phosphorylation/dephosphorylation at different stages in the assembly, maturation and trafficking of $\alpha 4\beta 2$ receptors is presented in Fig. 7.

Evidence that PKC has a dual action affecting $\alpha 4$ subunits associated with mature receptors at the cell surface and as intracellular subunits in the free and immature complexed states, resembles the effects of nicotine on these receptors. Nicotine has also been postulated to have a dual action, both at the cell surface and on immature $\alpha 4\beta 2$ oligomers in the endoplasmic reticulum. Several studies have supported the idea that nicotine acts intracellularly as a chaperone to promote subunit assembly (Kuryatov et al., 2005; Sallette et al., 2005), increase the transport of receptors from the endoplasmic reticulum to the plasma membrane (Darsow et al., 2005), and stabilize receptors at the cell surface (Peng et al., 1994; Wang et al., 1998; Kuryatov et al., 2005), albeit the cellular mechanism(s) involved have not been elucidated. Incubation of HEK293 cells with nicotine increases the number of receptors 2.5-fold and produces a 1.6-fold increased functional response (Sallette et al., 2005), similar to results obtained upon activation of PKC. These findings, in concert with studies demonstrating that both nicotine and PKC activation increase receptor function by increasing subunit assembly and translocation of the receptor to or near the cell surface, resulting in increased surface expression (Nashmi et al., 2003), suggests that at least some of the actions of nicotine may be mediated via activation of PKC.

Lastly, the present study indicates for the first time that $\beta 2$ subunits of mature $\alpha 4\beta 2$ receptors at the cell surface are phosphorylated in the basal state and can be further phosphorylated upon activation of both PKA and PKC. Although Exley et al. (2006) failed to identify any PKA phosphorylation sites on human $\beta 2$ subunits, predictions for putative phosphorylation sites indicate that a PKA, but not PKC consensus sequence is present in the large M3/M4 cytoplasmic loop of these subunits (<http://www.cbs.dtu.dk/services/NetPhosK/>). We have obtained preliminary data that phosphorylation occurs primarily on serine residues, but have not identified specific substrates or the functional consequences of $\beta 2$ subunit phosphorylation.

Current experiments are determining whether receptors normally expressed in brain tissue are phosphorylated at different maturational stages upon activation of PKA and PKC as those in studies from heterologous expression systems.

Acknowledgements

These studies were supported in part by a grant from the National Institute of Drug Abuse #DA14010 (to L.W.).

Abbreviations

2D	two-dimensional
ACh	acetylcholine
ECL	enhanced chemiluminescence
MOPS	3-(N-morpholino)propanesulfonic acid
NP-40	nonyl phenoxy polyethoxy ethanol
PBS	phosphate-buffered saline
PDBu	phorbol 12,13-dibutyrate
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
TBS	Tris-buffered saline
TLC	thin layer chromatography

References

- Almholt K, Arkhammar POG, Thastrup O, Tullin S. Simultaneous visualization of the translocation of protein kinase C α -green fluorescent protein hybrids and intracellular calcium concentrations. *Biochem J* 1999;337:211–218. [PubMed: 9882617]
- Boyle WJ, van der Geer P, Hunter T. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol* 1991;201:110–149. [PubMed: 1943760]

- Buisson B, Bertrand D. Chronic exposure to nicotine upregulates the human $\alpha 4\beta 2$ nicotinic acetylcholine receptor function. *J Neurosci* 2001;21:1819–1829. [PubMed: 11245666]
- Darsow T, Booker TK, Piña-Crespo JC, Heinemann SF. Exocytotic trafficking is required for nicotine-induced up-regulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *J Biol Chem* 2005;280:18311–18320. [PubMed: 15741168]
- Eaton JB, Peng JH, Schroeder KM, George AA, Fryer JD, Krishnan C, Buhlman L, Kuo YP, Steinlein O, Lukas RJ. Characterization of human $\alpha 4\beta 2$ -nicotinic acetylcholine receptors stably and heterologously expressed in native nicotinic receptor-null SH-EP1 human epithelial cells. *Mol Pharmacol* 2003;64:1283–1294. [PubMed: 14645658]
- Eilers H, Schaeffer E, Bickler PE, Forsayeth JR. Functional deactivation of the major neuronal nicotinic receptor caused by nicotine and a protein kinase C-dependent mechanism. *Mol Pharmacol* 1997;52:1105–1112. [PubMed: 9415721]
- Exley R, Moroni M, Sasdelli F, Houlihan LM, Lukas RJ, Sher E, Zwart R, Bermudez I. Chaperone protein 14-3-3 and protein kinase A increase the relative abundance of low agonist sensitivity human $\alpha 4\beta 2$ nicotinic acetylcholine receptors in *Xenopus* oocytes. *J Neurochem* 2006;98:876–885. [PubMed: 16787419]
- Fenster CP, Beckman ML, Parker JC, Sheffield EB, Whitworth TL, Quick MW, Lester RA. Regulation of $\alpha 4\beta 2$ nicotinic receptor desensitization by calcium and protein kinase C. *Mol Pharmacol* 1999;55:432–443. [PubMed: 10051526]
- Gopalakrishnan M, Molinari EJ, Sullivan JP. Regulation of human $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors by cholinergic channel ligands and second messenger pathways. *Mol Pharmacol* 1997;52:524–534. [PubMed: 9281615]
- Guo X, Wecker L. Identification of three cAMP-dependent protein kinase (PKA) phosphorylation sites within the major intracellular domain of neuronal nicotinic receptor $\beta 4$ subunits. *J Neurochem* 2002;82:439–447. [PubMed: 12124445]
- Hsu YN, Edwards SC, Wecker L. Nicotine enhances the cyclic AMP-dependent protein kinase-mediated phosphorylation of $\alpha 4$ subunits of neuronal nicotinic receptors. *J Neurochem* 1997;69:2427–2431. [PubMed: 9375675]
- Jeanclous EM, Lin L, Treuil MW, Rao J, DeCoster MA, Anand R. The chaperone protein 14-3-3 η interacts with the nicotinic acetylcholine receptor $\alpha 4$ subunit. Evidence for a dynamic role in subunit stabilization. *J Biol Chem* 2001;276:28281–28290. [PubMed: 11352901]
- Keller SH, Lindstrom J, Ellisman M, Taylor P. Adjacent basic amino acid residues recognized by the COP I complex and ubiquitination govern endoplasmic reticulum to cell surface trafficking of the nicotinic acetylcholine receptor α -subunit. *J Biol Chem* 2001;276:18384–18391. [PubMed: 11279119]
- Kuryatov A, Luo J, Cooper J, Lindstrom J. Nicotine acts as a pharmacological chaperone to up-regulate human $\alpha 4\beta 2$ acetylcholine receptors. *Mol Pharmacol* 2005;68:1839–1851. [PubMed: 16183856]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685. [PubMed: 5432063]
- Moroni M, Zwart R, Sher E, Cassels BK, Bermudez I. $\alpha 4\beta 2$ nicotinic receptors with high and low acetylcholine sensitivity: pharmacology, stoichiometry, and sensitivity to long-term exposure to nicotine. *Mol Pharmacol* 2006;70:755–768. [PubMed: 16720757]
- Nakayama H, Okuda H, Nakashima T. Phosphorylation of rat brain nicotinic acetylcholine receptor by cAMP-dependent protein kinase in vitro. *Brain Res Mol Brain Res* 1993;20:171–177. [PubMed: 8255179]
- Nashmi R, Dickinson ME, McKinney S, Jareb M, Labarca C, Fraser SE, Lester HA. Assembly of $\alpha 4\beta 2$ nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons. *J Neurosci* 2003;23:11554–11567. [PubMed: 14684858]
- Nelson ME, Kuryatov A, Choi CH, Zhou Y, Lindstrom J. Alternate stoichiometries of $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *Mol Pharmacol* 2003;63:332–341. [PubMed: 12527804]
- Nigam SK, Blobel G. Cyclic AMP-dependent protein kinase in canine pancreatic rough endoplasmic reticulum. *J Biol Chem* 1989;264:16927–16932. [PubMed: 2550466]

- Oancea E, Meyer T. Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell* 1998;95:307–318. [PubMed: 9814702]
- Pacheco MA, Pastoor TE, Wecker L. Phosphorylation of the $\alpha 4$ subunit of human $\alpha 4\beta 2$ nicotinic receptors: role of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). *Brain Res Mol Brain Res* 2003;114:65–72. [PubMed: 12782394]
- Peng X, Gerzanich V, Anand R, Whiting PJ, Lindstrom J. Nicotine-induced increase in neuronal nicotinic receptors results from a decrease in the rate of receptor turnover. *Mol Pharmacol* 1994;46:523–530. [PubMed: 7935334]
- Picciotto MR, Caldarone BJ, Brunzell DH, Zachariou V, Stevens TR, King SL. Neuronal nicotinic acetylcholine receptor subunit knockout mice: physiological and behavioral phenotypes and possible clinical implications. *Pharmacol Ther* 2001;92:89–108. [PubMed: 11916531]
- Pollock VV, Pastoor TE, Wecker L. Cyclic AMP-dependent protein kinase (PKA) phosphorylates ser362 and 467 and protein kinase C phosphorylates ser550 within the M3/M4 cytoplasmic domain of human nicotinic receptor $\alpha 4$ subunits. *J Neurochem* 2007;103:456–466. [PubMed: 17897355]
- Ren XQ, Cheng SB, Treuil MW, Mukherjee J, Rao J, Braunewell KH, Lindstrom JM, Anand R. Structural determinants of $\alpha 4\beta 2$ nicotinic acetylcholine receptor trafficking. *J Neurosci* 2005;25:6676–6686. [PubMed: 16014729]
- Rothhut B, Romano SJ, Vijayaraghavan S, Berg DK. Post-translational regulation of neuronal acetylcholine receptors stably expressed in a mouse fibroblast cell line. *J Neurobiol* 1996;29:115–125. [PubMed: 8748376]
- Sallette J, Pons S, Devillers-Thiery A, Soudant M, Prado de Carvalho L, Changeux JP, Corringer PJ. Nicotine upregulates its own receptors through enhanced intracellular maturation. *Neuron* 2005;46:595–607. [PubMed: 15944128]
- Shuck S, Honsho M, Ekroos K, Shevchenko A, Simons K. Resistance of cell membranes to different detergents. *Proc Natl Acad Sci U S A* 2003;100:5795–5800. [PubMed: 12721375]
- Shirai Y, Saito N. Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. *J Biochem* 2002;132:663–668. [PubMed: 12417013]
- Tapper AR, McKinney SL, Nashmi R, Schwarz J, Deshpande P, Labarca C, Whiteaker P, Marks MJ, Collins AC, Lester HA. Nicotine activation of $\alpha 4^*$ receptors: sufficient for reward, tolerance, and sensitization. *Science* 2004;306:1029–1032. [PubMed: 15528443]
- Vallejo YF, Buisson B, Bertrand D, Green WN. Chronic nicotine exposure upregulates nicotinic receptors by a novel mechanism. *J Neurosci* 2005;25:5563–5572. [PubMed: 15944384]
- Viseshakul N, Figl A, Lytle C, Cohen BN. The $\alpha 4$ subunit of rat $\alpha 4\beta 2$ nicotinic receptors is phosphorylated in vivo. *Brain Res Mol Brain Res* 1998;59:100–104. [PubMed: 9729306]
- Wang F, Nelson ME, Kuryatov A, Olale F, Cooper J, Keyser K, Lindstrom J. Chronic nicotine treatment up-regulates human $\alpha 3\beta 2$ but not $\alpha 3\beta 4$ acetylcholine receptors stably transfected in human embryonic kidney cells. *J Biol Chem* 1998;273:28721–28732. [PubMed: 9786868]
- Wang QJ, Fang TW, Fenick D, Garfield S, Bienfait B, Marquez VE, Blumberg PM. The lipophilicity of phorbol esters as a critical factor in determining the pattern of translocation of protein kinase C δ fused to green fluorescent protein. *J Biol Chem* 2000;275:12136–12146. [PubMed: 10766849]
- Wecker L, Guo X, Rycerz AM, Edwards SC. Cyclic AMP-dependent protein kinase (PKA) and protein kinase C phosphorylate sites in the amino acid sequence corresponding to the M3/M4 cytoplasmic domain of $\alpha 4$ neuronal nicotinic receptor subunits. *J Neurochem* 2001;76:711–720. [PubMed: 11158241]
- Whiteaker P, Cooper JF, Salminen O, Marks MJ, McClure-Begley TD, Brown RW, Collins AC, Lindstrom JM. Immunolabeling demonstrates the interdependence of mouse brain $\alpha 4$ and $\beta 2$ nicotinic acetylcholine receptor subunit expression. *J Comp Neurol* 2006;499:1016–1038. [PubMed: 17072836]
- Wu J, Kuo YP, George AA, Xu L, Hu J, Lukas RJ. β -myloid directly inhibits human $\alpha 4\beta 2$ -nicotinic acetylcholine receptors heterologously expressed in human SH-EP1 cells. *J Biol Chem* 2004;279:37842–37851. [PubMed: 15234980]
- Xu J, Zhu Y, Heinemann SF. Identification of sequence motifs that target neuronal nicotinic receptors to dendrites and axons. *J Neurosci* 2006;26:9780–9793. [PubMed: 16988049]

Zoli M, Le Novere N, Hill JA Jr, Changeux JP. Developmental regulation of nicotinic ACh receptor subunit mRNAs in the rat central and peripheral nervous systems. *J Neurosci* 1995;15:1912–1939. [PubMed: 7891142]

Fig. 1.

Immunoprecipitation of human nicotinic receptor $\alpha 4$ and $\beta 2$ subunits by mAb290 and mAb299. SH-EP1 cells were transfected with DNAs coding for $\alpha 4$ and $\beta 2$ subunits alone or in combination ($\alpha 4:\beta 2$, 2:3). [A] A representative immunoblot identifying $\alpha 4$ and $\beta 2$ subunits immunoprecipitated by mAb299 versus mAb290. Whole cell lysates were prepared and proteins were immunoprecipitated with either mAb299 (directed against $\alpha 4$ subunits) or mAb290 (directed against $\beta 2$ subunits). Samples were separated on SDS-PAGE gels and transferred to PVDF membranes. The membranes were cut just above the 59 kDa marker and the top half was probed for $\alpha 4$ with the H-133 antibody and the bottom half was probed for $\beta 2$ with the H-92 antibody; molecular weight standards are indicated. [B] Representative immunoblots from non-biotinylated and biotinylated proteins prior to and following endoglycosidase cleavage. Surface proteins were labeled with Sulfo-NHS-LC-Biotin 24 hours following transfections, whole cell lysates prepared, $\alpha 4\beta 2$ complexes immunoprecipitated with

mAb290, and samples separated on SDS-PAGE gels and transferred to PVDF membranes. Non-biotinylated samples were probed for $\alpha 4$ and $\beta 2$ subunits with H-133 and H-92, respectively, and biotinylated proteins were probed with peroxidase-conjugated extravidin. For each condition, samples were untreated (lane 1) or digested with the endoglycosidases Endo-H (lane 2) or PNGase F (lane 3). $\alpha 4_m$ and $\beta 2_m$ represent subunits immunoprecipitated from fully processed mature pentamers present mostly in the plasma membrane, and $\alpha 4_i$ and $\beta 2_i$ represent subunits immunoprecipitated from immature complexes located in the endoplasmic reticulum. [C] Sequential immunoprecipitations and detergent comparison. Following transfection, whole cell lysates were prepared in buffer containing either 2% Triton X-100 or 1% Lubrol. Subunits were immunoprecipitated sequentially with the indicated antibodies, and samples were separated on SDS-PAGE gels and transferred to PVDF membranes. The membranes were cut just above the 59 kDa marker and the top half was probed for $\alpha 4$ with the H-133 antibody and the bottom half was probed for $\beta 2$ with the H-92 antibody.

Fig. 2.

In vivo phosphorylation of immature and mature $\alpha 4$ subunits. SH-EP1 cells transfected with DNAs coding for $\alpha 4$ and $\beta 2$ subunits ($\alpha 4:\beta 2$, 2:3) were incubated with $^{32}\text{P}_i$ (1 or 2 mCi/ml) for 4 hours, the final 15 or 30 minutes in 10 μM forskolin or 200 nM PDBu, respectively. Whole cell lysates were prepared, $\alpha 4\beta 2$ complexes were immunoprecipitated with mAb290, and samples were separated on SDS-PAGE gels and transferred to PVDF membranes. Radioactivity was visualized by autoradiography by exposing the membranes to film prior to cutting them horizontally just above the 59 kDa marker. The top and bottom halves of the membranes were probed for subunit protein using H-133 for $\alpha 4$ and H-92 for $\beta 2$ subunit, respectively. A representative autoradiograph and immunoblot are shown depicting $\alpha 4_m$ and $\beta 2_m$ (subunits from fully processed pentamers) and $\alpha 4_i$ and $\beta 2_i$ (immature subunit complexes). Relative phosphorylation was determined as the ratio of the densitometric signals from the autoradiographs to the immunoblots. Bars represent the mean + s.e.m. from 4–6 experiments. The asterisks denote significant differences relative to corresponding basal values, $p < 0.05$.

Fig 3.

2D phosphopeptide maps of immature and mature $\alpha 4$ subunits following stimulation of PKA or PKC. SH-EP1 cells were transfected, incubated and proteins immunoprecipitated and separated as in Fig. 2. The [^{32}P]-labeled $\alpha 4_i$ and $\alpha 4_m$ bands were excised from the PVDF membranes, digested with trypsin, and spotted on TLC plates. Phosphopeptides were separated by electrophoresis in the horizontal dimension and ascending chromatography in the vertical dimension, and detected by autoradiography. Representative maps (from a total of 3 for each condition) from unstimulated cells (basal) or cells incubated in the presence of forskolin or PDBu are shown. Phosphopeptide clusters are grouped within circles.

[A] Phosphorylation of $\alpha 4$ subunits isolated from cells expressing only $\alpha 4$ subunits [B] Phosphorylation of $\alpha 4$ subunits isolated from cells expressing $\alpha 4$ and $\beta 2$ subunits **Fig 4.**

In vivo phosphorylation of homomeric $\alpha 4$ subunits. SH-EP1 cells were transfected with the DNA coding for either [A] $\alpha 4$ subunits only or [B] both $\alpha 4$ and $\beta 2$ subunits. Cells were incubated with ^{32}P i for 4 hours, the final 15 or 30 minutes in 10 μM forskolin or 200 nM PDBu, respectively, and whole cell lysates prepared as in Fig 2. [A] $\alpha 4$ subunits were immunoprecipitated with mAb299. [B] All $\alpha 4$ subunits assembled with $\beta 2$ subunits were removed by sequential immunoprecipitation with mAb290 and excess H-92. The remaining $\alpha 4$ subunits ($\alpha 4$) were immunoprecipitated with mAb299. Samples were separated on SDS-PAGE gels and transferred to PVDF membranes. Radioactivity was visualized by autoradiography, and subunit protein probed for $\alpha 4$ with H-133. A representative autoradiograph and immunoblot are shown. Relative phosphorylation was calculated as the ratio of densitometric signals from the autoradiographs to the immunoblots. Each bar represents the mean + s.e.m. from 4–6 experiments. The asterisks denote significant differences relative to corresponding basal values, $p < 0.05$. For 2D phosphopeptide maps, the ^{32}P -labeled $\alpha 4$ band was excised from the PVDF membranes, washed, digested with trypsin, and spotted on TLC plates as in Fig. 3. Phosphopeptide clusters are grouped within circles and labeled as in Fig. 3.

Fig. 5.

Effects of forskolin and PDBu on $\alpha 4\beta 2$ receptor density and function. [A] SH-EP1- $h\alpha 4\beta 2$ cells were incubated in the absence or presence of 10 μ M forskolin or 200 nM PDBu for 24 hours, membrane fractions prepared, and binding at 2.5 nM [³H]cytisine was determined. Bars represent group mean values + s.e.m. from 7 experiments. The asterisk denotes a significant difference from basal values, $p < 0.05$. [B] Responses of cells to varying concentrations of ACh. Membrane currents were measured using the perforated patch configuration of the whole-cell voltage clamp technique. The cell was held at -60 mV and membrane currents evoked by rapid application of ACh at the indicated concentrations. The horizontal bar indicates the time during the application of ACh (400 msec). A recording from a representative cell is shown on the left

and cumulative data depicting normalized peak ACh-induced inward currents as a function of ACh concentration on the right. Peak currents were normalized to currents measured in response to 3 mM ACh for each cell. Each point represents data obtained from 12–16 cells $\pm$ s.e.m. The solid line represents the results of a fit of the data to a 2-site Langmuir-Hill equation, while the dashed line shows a fit of the data to a 1-site equation. [C] Current-voltage relationship for ACh-evoked peak current amplitudes. A representative family of traces of ACh-activated current responses from a cell held at the indicated potentials and currents evoked using 2 mM ACh (400 msec). The mean peak ACh-induced inward currents as a function of holding potential are shown. Peak current amplitudes were normalized to the absolute peak measured at -60 mV for each cell in response to 2 mM ACh. Each point represents data from 7–9 cells $\pm$ s.e.m. [D] Modulation of ACh-evoked current density in SH-EP1- $h\alpha 4\beta 2$ cells following activation of PKA or PKC. Cells were incubated in the absence or presence of 10 μ M forskolin or 200 nM PDBu for 24 hours, washed, and membrane currents in response to 2 mM ACh were measured using the perforated patch configuration of the whole-cell voltage clamp technique; cells were held at -60 mV. Bar graphs depict group mean values $\pm$ s.e.m. of current densities (normalized to controls). The control ACh current density was -103 ± 5.5 pA/pF; $n=9$ cells from each group. The asterisk denotes a significant difference from basal values, $p<0.05$.

Fig 6.

Modulation of ACh-evoked currents in SH-EP1- $\alpha 4\beta 2$ cells by PDBu. Cells were incubated in the absence or presence of 200 nM PDBu or 100 nM Ro-31-8220 alone or in combination for different periods of time, and membrane currents in response to 2 mM ACh were measured following washing using the perforated patch configuration of the whole-cell voltage clamp technique; cells were held at -60 mV. The horizontal bars indicate the time of ACh application (1.5 sec). Representative current traces and bar graphs depicting group mean values $\pm$ s.e.m. of current densities (normalized to controls) are shown. [A] Cells were incubated for 24 hours with the indicated compounds and washed for 10 minutes prior to recording. The control ACh current density was -71.6 ± 5.1 pA/pF ($n=54$). The sample size (n) for the other groups were: 17 for DMSO, Ro-31-8220 and Ro + PDBu; and 29 for PDBu alone. [B] Cells were incubated for 1 hour with the indicated compounds followed by an extensive 10 minute washout prior to recording. The control ACh current density was -57.3 ± 3.7 pA/pF ($n=47$). The sample sizes (n) for the other groups were 13 for DMSO and 25 for PDBu. [C] Cells were incubated for 1 hour with the indicated compounds followed by washout and 23 hours of incubation in control media prior to recording. The control ACh current density was -74.1 ± 4.6 pA/pF ($n=61$). The sample sizes (n) for the other groups were: 16 for DMSO; 13 for Ro-31-8220; 28 for PDBu; 19 for Ro + PDBu. The asterisks denote significant differences from control ($p < 0.05$).

Fig. 7.

Putative sites of action of PKA and PKC on $\alpha 4\beta 2$ receptors. The schematic depicts the stages in the assembly, maturation and trafficking of $\alpha 4\beta 2$ receptors during which subunits may be phosphorylated (or dephosphorylated). Immature $\alpha 4$ subunits ($\alpha 4_i$) are shown representing free, unassembled subunits and immature subunit complexes containing high mannose in the endoplasmic reticulum. Mature $\alpha 4$ subunits ($\alpha 4_m$) are shown representing fully processed mature forms with complex carbohydrates associated with the Golgi and plasma membrane. Results from phosphorylation and differential immunoprecipitation experiments indicate that free $\alpha 4$ subunits are phosphorylated by both PKA and PKC, the former on Ser362 and Ser467, and the latter on two as yet unidentified serine residues (within C5 and C6). Phosphorylation at these sites promotes the assembly of subunits, followed sequentially by the PKC-mediated phosphorylation of Ser550. Although the schematic depicts all immature complexes phosphorylated by PKC, these complexes exist in at least two conformations, a juvenile form which can be immunoprecipitated with mAb299 and a late developmental form, which can be immunoprecipitated by mAb290 (Salette et al., 2005). It is possible that phosphorylation by PKC occurs only on one form, and that provides the signal for the exiting of the receptor from the endoplasmic reticulum. Maturation of the receptor in the Golgi appears to involve the dephosphorylation of several residues, perhaps one of which enables the pentamer to exit the Golgi and insert into the plasma membrane. $\alpha 4$ subunits associated with the plasma membrane

may be further phosphorylated on Ser550 by PKC, perhaps affecting receptor function or stabilization. $\beta 2$ subunits can also be phosphorylated by PKA and PKC, but only when are part of the membrane-associated pentamer.