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CYCLIC AMP-DEPENDENT PROTEIN KINASE A AND PROTEIN KINASE C PHOSPHORYLATE $\alpha4\beta2$ NICOTINIC RECEPTOR SUBUNITS AT DISTINCT STAGES OF RECEPTOR FORMATION AND MATURATION

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Abstract—Neuronal nicotinic receptor $\alpha4$ subunits associated with nicotinic $\alpha4\beta2$ receptors are phosphorylated by cyclic AMP-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 $\alpha4$ and/or $\beta2$ subunits were incubated with ^{32}Pi, and PKA or PKC was activated by forskolin or phorbol 12,13-dibutyrate, respectively. Immunoprecipitation and immunoblotting of proteins from cells expressing $\alpha4\beta2$ receptors or only $\alpha4$ subunits were used to identify free $\alpha4$ subunits, and $\alpha4$ subunits present in immature $\alpha4\beta2$ complexes and mature $\alpha4\beta2$ pentamers containing complex carbohydrates. In the absence of kinase activation, phosphorylation of $\alpha4$ subunits associated with mature pentamers was three times higher than subunits associated with immature complexes. PKA and PKC activation increased phosphorylation of free $\alpha4$ subunits on different serine residues; only PKC activation phosphorylated subunits associated with mature $\alpha4\beta2$ 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 $\beta2$ subunits associated with mature $\alpha4\beta2$ receptors. Results indicate that activation of PKA and PKC leads to the phosphorylation $\alpha4\beta2$ receptors at different stages of receptor formation and maturation and has differential effects on the expression and function of human $\alpha4\beta2$ receptors.

Key words: protein kinase A, protein kinase C, neuronal nicotinic receptor, phosphorylation, receptor formation, receptor function.

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Abbreviations: ACh, acetylcholine; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; Endo-H, endoglycosidase-H; PAGE, polyacrylamide gel; PBS, phosphate-buffered saline; PDBu, phorbol 12,13-dibutyrate; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PNGase F, peptide N-glycanase F; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TLC, thin-layer chromatography; 2D, two-dimensional.

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Neuronal nicotinic acetylcholine (ACh) receptors composed of $\alpha4$ and $\beta2$ subunits are widely distributed throughout the brain and represent the most abundant nicotinic receptor type in the brain (Zoli et al., 1995). These $\alpha4\beta2$ 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 $\alpha4\beta2$ receptors are regulated post-translationally through phosphorylation/dephosphorylation mechanisms involving both cyclic AMP-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 $\alpha4$ and $\beta2$ subunit cDNAs indicated that activation of PKA by forskolin increased the number of cell surface receptors twofold (Rothhut et al., 1996). Furthermore, recent findings using rat $\alpha4\beta2$ receptors expressed in tsA201 cells have suggested that this increase may be attributed to the PKA-dependent phosphorylation of $\alpha4$ 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 $\alpha4\beta2$ 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 $\alpha4$ subunit phosphorylation in mediating this action was not determined.

The phosphorylation of $\alpha4$ 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 $\alpha4$ subunit protein during different stages in the maturation of the $\alpha4\beta2$ pentamer. Pollock et al. (2007) have shown that two forms of $\alpha4$ subunit protein can be immunoprecipitated from SH-EP1 cells transiently transfected with the DNAs coding for human $\alpha4$ and $\beta2$ 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 α4 protein was unaltered following activation of PKA, but increased following activation of PKC. Based on studies suggesting that the lower molecular weight α4 protein represents immature α4 subunit species while the higher molecular weight protein represents subunits isolated from mature $\alpha4\beta2$ pentamers (Vallejo et al., 2005; Sallette et al., 2005), results suggest that PKA activation may increase phosphorylation of α4 subunits prior to formation of mature receptors, whereas PKC activation may have a dual action, phosphorylating α4 subunits both prior to after formation of the mature receptor. Thus, these studies investigated the phosphorylation of α4 subunits by PKA and PKC at distinct stage(s) in the assembly and maturation of $\alpha4\beta2$ receptors, and determined whether activation of PKA and PKC has 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 α4 subunit and mAb290 (N8533) raised against the extracellular loop of the rat nicotinic receptor β2 subunit, both of which cross react with the human subunits (Sigma-Aldrich Co., St. Louis, MO, USA). 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 α4 subunit, H-92 (sc-11372), a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 342–433 of the human β2 subunit, and goat anti-rabbit IgG horseradish peroxidase–conjugated secondary antibody (sc-2030), purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Sulfo-NHS-LC-biotin was purchased from Pierce (Rockford, IL, USA), endoglycosidase-H (Endo-H) and peptide N-glycanase F (PNGase F) were bought from New England Biolabs (Beverly, MA, USA), and Lipofectamine™ 2000 and cell growth media were purchased from Invitrogen Corporation (Carlsbad, CA, USA). 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, USA). [^{32}Pi] and [^{3}H]cytisine were purchased from Perkin Elmer Life and Analytical Sciences Inc. (Boston, MA, USA), polyvinylidene fluoride (PVDF) membranes (Immobilon-P) from Millipore Corporation (Bedford, MA, USA), and XAR film from Eastman Kodak Co. (Rochester, NY, USA). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA), basic electrophoresis chemicals from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and sequencing grade trypsin from Roche Diagnostics Corporation (Indianapolis, IN, USA). Human α4 and β2 ACh receptor subunit cDNA clones in pSP64 (kindly provided by Dr. Jon Lindstrom, Department of Neuroscience, University of Pennsylvania, Philadelphia, PA, USA) 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-*h*$\alpha4\beta2$ cells were kindly provided by Dr. Ron Lukas (Barrow Neurological Institute, Phoenix, AZ, USA).

Transfection, ^{32}Pi 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 μg/ml streptomycin, and 50 μg/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 α4 only, β2 only, or a mixture (α:β, 2:3) for 2–3 h at 37 °C and for an additional 3–4 h at 32 °C. The Lipofectamine was removed and cells were incubated with growth media lacking antibiotics for an additional 18–20 h at 32 °C, for a total incubation time of 24 h.

To label endogenous ATP stores, cells were incubated with ^{32}Pi (1 or 2 mCi/ml) for 4 h 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 was added during the final 15 or 30 min, 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×) with ice-cold phosphate-buffered saline (PBS) followed by incubation for 30 min 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 μg/ml pepstatin A, 10 μg/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 min at 4 °C on a rotator, and were centrifuged at 100,000×*g* for 10 min; the supernatants were withdrawn and transferred to tubes for further experimentation.

Immunoprecipitations

α4 Subunit protein was immunoprecipitated from whole cell lysates using both the anti-α4 monoclonal antibody mAb299 and the anti-β2 monoclonal antibody mAb290. Studies have shown that mAb299 immunoprecipitates subunits associated only with immature receptors from transfected HEK293 cells expressing $\alpha4\beta2$ 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 μl protein-G sepharose for 30 min at 4 °C. The samples were incubated with primary antibody (5 μg of mAb299 or mAb290) overnight with rocking at 4 °C, followed by rocking with 30 μl protein-G sepharose for 3–4 h. The protein/antibody/bead complexes were washed five times with 50 mM 3-(N-morpholino) propanesulfonic acid 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 2000×*g* for 5 min 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 2000×*g* for 5 min. The supernatants were reincubated with antibody and protein-G sepharose and the protein/antibody/bead complexes were processed as described. This procedure was repeated two more times for a total of four sequential immunoprecipitations.

To isolate α4 subunits that were not associated with β2 subunits from cells expressing $\alpha4\beta2$ receptors, all $\alpha4\beta2$ complexes were immunoprecipitated from pre-cleared cell lysates by sequential incubation with mAb290 and excess (10 μg) H-92, a polyclonal anti-β2 antibody that is not conformation or assembly dependent. Samples were incubated with mAb290 and protein-G sepharose