The Chaperone Protein 14-3-3eta Interacts with the Nicotinic Acetylcholine Receptor α4 Subunit: Evidence for a Dynamic Role in Subunit Stabilization

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SUMMARY

Using the large cytoplasmic domain of the nicotinic acetylcholine receptor (AChR) α4 subunit as a bait in the yeast two-hybrid system, we isolated a cytosolic protein, 14-3-3η, known to directly interact with neuronal AChRs. 14-3-3η is a member of a family of proteins that function as regulatory or chaperone/scaffolding/adaptor proteins. 14-3-3η interacted with the recombinant α4 subunit alone in tsA 201 cells following activation of cAMP-dependent protein kinase (PKA) by forskolin. The interaction of 14-3-3η with recombinant α4 subunits was abolished when serine 441 of the α4 subunit is mutated to alanine (α4S441A). The surface levels of recombinant wild-type α4β2 AChRs were ~2-fold higher than those of mutant α4S441Aβ2 AChRs. The interaction significantly increased the steady state levels of the α4 subunit and α4β2 AChRs but not that of the mutant α4S441A subunit or mutant α4S441Aβ2 AChRs. The EC₅₀’s for activation by ACh were not significantly different for α4β2 AChRs and α4S441Aβ2 AChRs coexpressed with 14-3-3η in oocytes following treatment with forskolin. 14-3-3 coimmunopurified with native α4 AChRs from brain. These results support a role for 14-3-3 in dynamically regulating the expression levels of α4β2 AChRs through its interaction with the α4 subunit.
INTRODUCTION

Neuronal nicotinic acetylcholine receptors (AChR)\(^1\) are a family of ligand-gated, cation-selective, homo- or heteropentameric ion channels expressed in the peripheral and central nervous system (1,2). A multitude of neuronal AChR subtypes assembled from different combinations of \(\alpha_2-\alpha_9\) and \(\beta_2-\beta_4\) subunits have been identified (3,4). Of these, the \(\alpha_4\beta_2\) AChR is widely expressed in the CNS and represents >80% of the high-affinity \([\text{^3}H]\) nicotine-binding sites in mammalian brain (5). Our understanding of their physiological roles comes most recently from gene knock-out studies in mice. Mice in which the \(\alpha_4\) subunit gene has been deleted lack \([\text{^3}H]\) nicotine or \([\text{^3}H]\) epibatidine binding sites in their brain and exhibit reduced antinociceptive effects of nicotine (6). Mice in which the \(\beta_2\) subunit gene has been deleted also show little \([\text{^3}H]\) nicotine binding in their brains, lose their sensitivity to nicotine in passive avoidance tasks (7), and show attenuated self-administration of nicotine (8) suggesting that \(\alpha_4\beta_2\) AChRs have a role in mediating addiction to nicotine. The normal and pathophysiological functions mediated by \(\alpha_4\beta_2\) AChRs are of significant importance to human health. Some inherited forms of epilepsy, such as the autosomal dominant nocturnal frontal lobe epilepsies, are caused by \(\alpha_4\beta_2\) AChRs harboring at least two separate mutations within their \(\alpha_4\) subunit (9-12). Most recently, \(\alpha_4\beta_2\) AChRs, among other \(\beta_2\) subunit-containing AChRs, have been

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\(^1\) Ab, antibody; AChR, nicotinic acetylcholine receptor; ACh, acetylcholine; BSA, bovine serum albumin; CRE, cAMP response element; CREB, CRE binding protein; DMEM, Dulbecco's modified Eagle's medium; EC\(_{50}\), half-maximal effective concentration of agonist; ERK, extracellular-signal regulated kinase; HEPES, N-[2-hydroxyethyl]piperazine-N'-2-ethane sulfonic acid; HRP, horseradish peroxidase; IBMX, 3-iso-butyl-1-methylxanthine; mAb, monoclonal antibody; NGS, normal goat serum; \(n_H\), hill coefficient; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; PKA, protein kinase A, PKC,
implicated in neuronal survival during ageing, as surmised from the neurodegeneration observed in β2-subunit knock-out mice (13).

The α4 subunit, like the other AChR subunits, consists of an extracellular N-terminal domain, followed by three transmembrane domains (M1-M3), a large cytoplasmic domain, a fourth transmembrane domain (M4), and a short extracellular C-terminus. The large cytoplasmic domain is highly divergent among the various subunits and this sequence divergence presumably provides the diversity necessary for different AChR subtypes to directly interact with cytosolic proteins of different function. To identify such proteins associated with α4β2 AChRs, we used the large cytoplasmic domain of the α4 subunit as a bait to screen a mouse brain cDNA yeast two-hybrid library. Here we describe the isolation of a known protein termed 14-3-3η. The 14-3-3 proteins family consists of seven isoforms (β,γ,η,ξ,ε,τ,σ) that function as intracellular regulators or chaperone/scaffolding/adaptor proteins in diverse cellular functions (14). The binding of 14-3-3 to most of their protein targets are mediated by a phosphoserine or phosphothreonine residue within a consensus binding site motif or within sequences closely resembling it (15).

We found that 14-3-3η interacted with the recombinant AChR α4 subunits alone following activation of PKA. The interaction was mediated by serine 441 of the α4 subunit within a motif similar to a known consensus binding site motif for 14-3-3 protein kinase C; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;
proteins. The interaction significantly increased the steady state levels of the α4 subunit alone and α4β2 AChRs. The surface levels of recombinant wild-type α4β2 AChRs were ~2-fold higher than those of mutant α4S441Aβ2 AChRs. 14-3-3 coimmunopurified with native α4 AChRs from brain suggesting its interaction with native α4 AChRs is physiologically relevant. These results support a possible role for 14-3-3 in dynamically regulating the steady state levels of α4β2 AChRs through its interaction with the α4 subunit in the ER/Golgi compartments, following activation of PKA.
EXPERIMENTAL PROCEDURES

Constructs. The rat α4 and β2 AChR subunit cDNA clones were generously provided by Dr. Stephen Heinemann (Salk Institute, San Diego, CA). All constructs were made by the polymerase chain reaction (PCR) using appropriate pairs of forward and reverse synthetic oligonucleotide primers (Life Technologies, Inc., Bethesda, MD) and Pfu Turbo (Stratagene, Inc., San Diego, CA). All DNA sequence analysis was done using the ThermoSequenase radiolabeled terminator cycle sequencing Kit (Amersham). For all primers, the restriction enzyme sites are shown in italics. The cDNA sequence corresponding to the large cytoplasmic domain (amino acids 302-561) of the rat α4 subunit was amplified using the forward primer 5'-GGG GAA TTC GTG CAC CAC CGC TCG CCA CGC-3' and the reverse primer 5'-CCC GGA TCC TCA CTT CAC CGA GAA GT C AG TGT C-3' by PCR and subcloned into the EcoRI-BamHI sites of the vector pLexA (Clontech Laboratories, Inc., Palo Alto, CA) to form the α4 bait. The nested deletions of the α4 cytoplasmic domain were generated by PCR using the forward primer 5'-GGG GAA TTC GTG CAC CAC CGC TCG CCA CGC-3' and nested reverse primers 5'-GGG GGA TCC TCA GGT GCC TCC CGC CTT GAG CAC-3'; 5'-GGG GGA TCC TCA CAG GGA GGT CGG GGA GCT GGT-3'; 5'-GGG GGA TCC TCA GTT GTC TTT GAC CAC AGA GGG-3' and were subsequently subcloned into the EcoRI-BamHI sites of the pLexA. The mutated subunit α4^{S441A} was generated by mutagenesis using the following two primers: 5'-C AAA GCC by guest on March 22, 2020http://www.jbc.org/Downloaded from
AGG TCC CTG GCT GTC CAG CAT GTG CCC-3’ and 5’-GGG CAC ATG CTG GAC AGC CAG GGA CCT GGC TTT G-3’ in conjunction the QuikChange site-directed mutagenesis kit (Stratagene Inc., San Diego, CA). The mouse 14-3-3\(\eta\), the rat \(\alpha_4\) subunit, and the rat \(\beta_2\) subunit cDNAs were generated by amplification of the full clone by PCR using forward primer 5’-GGG AAT TCG CCA CCA TGG GGG ATC GAG AGC AG-3’ and reverse primer 5’-GGG TCT AGA TCA GTG GCC TTC TCC TGC TTC TTC-3’ for 14-3-3\(\eta\); forward primer 5’-GGG AAT TCG CCA CCA TGG CCA ATT CGG GCC CCG GG-3’ and reverse primer 5’-GGG TCT AGA TCA GCA AGC AGC CAG CCA GGG AGG-3’ for the \(\alpha_4\) subunit ; and forward primer 5’-GGG AAT TCG CCA CCA TGC TGG CTT GCA TGG CCG GG-3’ and reverse primer 5’-GGG TCT AGA TCA CTT GGA GCT GGG AGC TGA GTG-3’ for the \(\beta_2\) subunit. The amplified cDNAs were ligated into the EcoRI-XbaI sites of the mammalian cell expression vector pEF6/myc-His A (Invitrogen, Carlsbad, CA).

**Yeast two-hybrid library screen.** Yeast two-hybrid screens were carried out according to a standard protocol (Clontech Laboratories Inc., Palo Alto, CA). The \(\alpha_4\) bait plasmid pLexA and the p8op-LacZ reporter gene plasmid were first transformed in EGY48 yeast cells followed by transformation of the library of brain cDNA plasmids. Approximately 2x10^6 yeast cells cotransformed with the bait and cDNAs from a premade mouse brain cDNA Matchmaker LexA library (Clontech Laboratories Inc., Palo Alto, CA) were screened. Positive clones were selected for their ability to grow on plates lacking leucine, tryptophan, histidine, and uracil and assayed for \(\beta\)-galactosidase activity on media supplemented with X-gal. Plasmids containing the brain cDNAs were isolated from
positive yeast cells and their nucleotide sequences determined by manual DNA sequencing using the ThermoSequenase radiolabeled terminator cycle Kit (Amersham Pharmacia Biotech., Inc., Piscataway, NJ). Seven clones of 14-3-3η that interacted with the α4 bait were characterized. These clones contained slightly different cDNA sizes but all had the full-length cDNA of 14-3-3η as determined from limited sequence analysis of their 5′ ends. One of these clones containing the full-length 14-3-3η subunit was used in all subsequent work.

**Antibodies.** The anti-14-3-3 mouse monoclonal antibody that crossreacts with multiple isoforms of 14-3-3 and the anti-β2 subunit that crossreacts with β2 subunits on immunoblots was obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Protein G affinity-purified anti-α4 subunit (299) and anti-β2 subunit (295) mAbs were generously provided by Dr. Lindstrom (U. Pennsylvania, Philadelphia, PA). The goat anti-mouse, and anti-rat horseradish peroxidase conjugated Abs were obtained from Pierce, Rockford, IL. mAbs were coupled to Actigel ALD beads at a concentration of 0.5 mg/ml of gel using the manufacturer's instructions (Sterogene Bioseparations Inc., Carlsbad, CA).

**Expression of recombinant AChR subunits in human embryonic kidney tsA201 cells.**

Human tsA201 cells (16), a derivative of the human embryonic kidney (HEK) cell line 293 were cultured at 37°C in 6 well plates in DMEM (Life Technologies Inc., Bethesda, MD) supplemented with 10% fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Cells were transfected using lipofectamine 2000 (Life Technologies Inc.,
Bethesda, MD) at 90% confluency (~10^6 cells/well) with various combinations of cDNAs as per the manufacturer's instructions and utilized after ~48h. The cDNAs were cloned into the vector pEF6/myc-His but lacked the myc-His tag because of the presence of the endogeneous stop codon present in each cloned cDNA. AChR subunit assembly was found to be more efficient at 30°C than at 37°C as previously described (17) and hence the experiments were performed with cells incubated at 30°C following transfection.

**Expression and analysis of the α4 subunit.** To study the effect of 14-3-3η on the α4 subunit alone, tsA201 cells seeded (400,000 per well) in 12 wells, were incubated at 37°C. The next day the cells were cotransfected with α4 or α4S441A with or without 14-3-3η and incubated at 30°C (the DNA concentrations and ratios were kept constant by using the pEF6A vector DNA). The transfected cells were treated 24 h with or without forskolin (10µM). After washing once with ice cold PBS, the cells were solubilized in 500µl of the following lysis buffer: 50mM NaCl, 30mM triethanolamine, pH 7.5, 5mM EGTA, 5mM EDTA, 1mM benzamidine, 5µg/ml aprotinin, 5µg/ml leupeptin, 5µg/ml pepstatin and 2% NP-40. After shaking for 3 hours at 4°C, the lysates were centrifugated for 15 min. at 18,000xg and 20µl of the supernatant were analyzed by SDS-PAGE.

**Immunoisolation of recombinant AChRs in tsA201 cells.** tsA201 cells were washed twice with ice cold PBS containing 50mM NaF and 1mM sodium orthovanadate and lysed in 1 ml of lysis buffer (50mM NaCl, 30mM triethanolamine, pH 7.5, 5mM EGTA, 5mM EDTA, 50mM NaF, 1mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 2mM sodium vanadate, 10mM p-nitrophenylphosphate, 25µg/ml aprotinin, 25µg/ml
leupeptin, 25µg/ml pepstatin, 0.3µM okadaic acid, 1mM sodium tetrathionate, 1 mM N-ethylmaleimide, 50 µM phenylarsine oxide and 1% NP-40) agitated vigorously for 2 h at 4°C. After centrifugation at 18, 000 xg for 15 min the clear supernatant from each sample (~1ml) was incubated with 10µl of mAb-coupled beads (that were preblocked with 5% non-fat milk for 30 min) for 24 h. The beads were then washed 8 times with ~800µl of solubilization buffer and eluted with sample buffer (lacking β-mercaptoethanol to avoid reduction of the disulphide linkage of the IgG chains) at 60°C for 30 min and then β-mercaptoethanol was added to the eluted samples that were then boiled for an additional 5 min prior to analysis by SDS-PAGE.

Immunoisolation of native AChRs from rat brain. Frozen rat brains were homogenized in 10 vol of homogenization buffer (50mM NaCl, 30mM triethanolamine, pH 7.5, 5mM EGTA, 5mM EDTA, 50mM NaF, 1mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 2mM sodium vanadate, 10mM p-nitrophenylphosphate) using a homogenizer (OMNI International, Warrenton, VA). The homogenate was centrifuged at 100, 000 xg in a Beckmann 50.2 Ti rotor for 30 min at 4°C. The membrane pellet was further briefly homogenized and then extracted with 3 volumes of a solubilization buffer (homogenization buffer containing 1% NP-40 and 25µg/ml aprotinin, 25µg/ml leupeptin, 25µg/ml pepstatin, 0.1µM okadaic acid, 1mM sodium tetrathionate, 1mM N-ethylmaleimide, 50µM phenylarsine oxide) for 2 h at 4°C. The clear supernatant obtained after centrifugation of the pellet at 18, 000xg for 30 min was used for all subsequent immunoisolation procedures. Detergent-solubilized brain extracts (typically 10 ml) thus obtained were incubated with approximately 25µl of mAb-coupled Actigel ALD bead
(that were preblocked with 5% non-fat milk for 30 min) at 4°C for 72 h. In initial experiments, to ensure that the binding observed was specific, we first determined the number of successive washes of the mAb-beads necessary for the complete removal of unbound 14-3-3 proteins which are abundant in brain extracts and many of whose isoforms crossreact with the anti-14-3-3 mAb used in the immunoblotting experiments. The beads thus were typically washed 10 times with ~800 µl of solubilization buffer and eluted with sample buffer (lacking β-mercaptoethanol to avoid reduction of the disulphide linkage of the IgG chains) at 60°C for 30 min and then β-mercaptoethanol was added to the eluted samples prior to analysis by SDS-PAGE.

**Immunoblot analysis.** The proteins bound to the Ab-beads were eluted with protein sample buffer and fractionated by SDS-PAGE. The proteins were electroblotted onto polyvinylidene difluoride membrane (IMMUN-BLOT; Bio-Rad Laboratories, Hercules, CA) and the membranes incubated with diluted (typically 1:200 to 1:1000) primary Abs in phosphate-buffered saline solution containing 0.1% Tween and 5% non-fat milk. The binding of the primary mAbs was detected using appropriate secondary Abs conjugated to horseradish peroxidase in conjunction with a chemiluminescence detection kit (SuperSignal, Pierce). To reduce nonspecific binding, the blots were typically cut in half and the top half probed with the anti-α4 subunit mAb and the bottom half with the anti-14-3-3 mAb thus eliminating the need for sequential reprobing of the blots.

**Enzyme-linked immunoassay for cell surface AChRs.** Cell surface α4β2 AChRs were measured as previously described (17). Briefly, 48 h after transfection, tsA201 cells
plated in 12 well plates (0.5x10^6 cells/well) were washed once in PBS, then blocked with PBS containing 3% BSA and the cells were incubated for 1h with an anti-β2 subunit mAb (295) in PBS containing 3% BSA at room temperature. After four washes with PBS the cells were fixed with formaldehyde (3%) for 10 min, washed three times with PBS and blocked again for 10 min. The cells were then incubated with horseradish peroxidase-conjugated goat anti-rat secondary Ab for 1 h in the presence of 3% BSA, washed six times with PBS, and incubated with 300µl of the HRP substrate 3, 3', 5, 5'-tetramethylbenzidine (Sigma) for 1 h. The absorbance of the supernatant was then measured at 655nm in a Beckmann spectrophotometer.

Expression in Xenopus oocytes. cDNAs were subcloned into the vector pSP64T (Invitrogen) with a modified polylinker. cRNAs from linearized cDNA templates were synthesized in vitro using SP6 RNA polymerase in conjunction with reagents from the mMessage mMachine kit (Ambion, Austin, TX). Xenopus oocytes were prepared for injection as previously described (18). Oocytes were injected with 20ng of cRNAs for the α4 and β2 subunits and 40ng of 14-3-3η per oocyte and incubated for 3-7 days at 16-18°C in 50% L-15 medium (Life Technologies Inc.) containing 10mM HEPES buffer, pH 7.5.

Electrophysiological recordings. Currents were measured using standard two-microelectrode voltage-clamp amplifier (Oocyte Clamp OC-725C) as previously described (18). Electrodes were filled with 3M KCl and had resistances of 1.0-2.0 mega-ohm for the voltage electrode and 0.5-0.1 mega-ohm for the current electrode. All
records were digitized at 200 Hz with MacLab software and hardware (AD Instruments). Data was analyzed using KALIEDAGRAPh. The recording chamber was perfused at a flow rate of 10ml/min with ND-96 solution (96mM NaCl, 5mM KCl, 1.8mM CaCl₂, 1mM MgCl₂, 5 mM HEPES, pH 7.6).

**Immunohistochemistry.** Cells were fixed with an ice cold mixture of 50% methanol:50% acetone for 5 min, washed 3 times with 2ml of PBS, and blocked using PBS containing 2% BSA for 30 min. Cells were then incubated with diluted primary anti-α4 mAb (1/2000 dilution) in PBS containing 4% NGS with gentle shaking for 1 h at 4°C. Cells were washed 3 times for 15 min per wash in PBS, incubated with diluted (1/1000) fluorophore-conjugated anti-rat secondary Ab in PBS containing 4% NGS for 1 h at room temperature and fixed again with an ice cold mixture of 50% methanol:50% acetone for 5 min and washed 3 times with PBS. Cells were then incubated with diluted primary anti-14-3-3 mAb (1/200 dilution) in PBS containing 4% NGS with gentle shaking for 1 h at 4°C. Cells were washed 3 times for 15 min per wash in PBS and then incubated with diluted (1/1000) fluorophore-conjugated anti-mouse secondary Ab in PBS containing 4% NGS for 1 h at room temperature. The cells were then washed 3 for 15 min periods in PBS containing Triton X-100 and then used for immunofluorescence microscopy. Nontransfected cells were processed in parallel as controls for nonspecific staining. The secondary Abs used were goat anti-rat Alexa Fluor 488 conjugated Ab (Cat# A-11006) and the goat anti-mouse Alexa Fluor 546 conjugated Ab (Cat# A-11030) from Molecular Probes, Eugene, OR.
Confocal microscopy. Confocal microscopy was accomplished using a Noran Instruments Odyssey XL confocal laser scanning microscope (Noran Instruments, Middleton, WI). Cells were scanned using 1 µm steps in the Z-axis, alternating between FITC and rhodamine filters. The resulting confocal images were captured on Silicon Graphics workstations. Images were pseudocolored and processed using Adobe Photoshop software.
RESULTS

14-3-3η interacts with the large cytoplasmic domain of the α4 subunit in the yeast two-hybrid system

The large cytoplasmic domain corresponding to amino acids 302-561 of the rat AChR α4 subunit was used as a bait to screen ~2x10⁶ clones of a mouse brain cDNA LexA yeast two-hybrid library. The large cytoplasmic domain extends from the third to the fourth transmembrane domain of the AChR α4 subunit. Multiple clones of 14-3-3η that interacted with the α4 bait were obtained. A full-length clone of 14-3-3η was chosen for further characterization.

To delineate the site at which 14-3-3η interacts with the AChR α4 subunit cytoplasmic domain, a series of C-terminal nested deletions of the cytoplasmic loop was created as LexA fusion protein baits and tested for their ability to interact with the 14-3-3η clone in the yeast two-hybrid system. The interaction was determined by both the ability of transformed yeast cells to grow on media lacking leucine, tryptophan, histidine, and uracil and by their ability to turn blue on media supplemented with X-gal. As controls, the LexA protein alone was used as a bait. We mapped the interaction of 14-3-3η to residues 413 to 450 (Figure 1A). A putative motif, RSXSXP, in which the underlined serine residue is phosphorylated, has been previously demonstrated to be important for the binding of 14-3-3 proteins to some of its target proteins. A sequence that closely resembles this motif, RSLSVQ, occurs within the region 413-450 that was
found to be essential for the interaction of 14-3-3 with the α4 cytoplasmic domain bait in the yeast two-hybrid system.

Based on the demonstration that the interaction of 14-3-3 with target proteins is mediated by the recognition of a phosphoserine, we mutated serine 441 to alanine within this consensus binding site motif in the α4 cytoplasmic domain. Mutating serine 441 to alanine nearly completely abolished interaction of 14-3-3η with the α4 cytoplasmic domain bait in the yeast two-hybrid system (Figure 1B). These results suggested that the high-affinity binding of 14-3-3η to the α4 cytoplasmic domain is mediated by serine 441 within a specific consensus binding site motif.

14-3-3η interacts with recombinant α4 subunits in tsA 201 cells

To directly test if 14-3-3η could interact with full-length unassembled α4 subunits alone, we transfected tsA 201 cells with the α4 subunit cDNA. Because it has been previously demonstrated that phosphorylation of a consensus binding site motif for 14-3-3 greatly increases its affinity for the site, we also tested if activation of kinases would increase the interaction of 14-3-3η with the recombinant α4 subunit. We treated transfected cells coexpressing 14-3-3η and recombinant α4 subunits with forskolin (50μM) plus IBMX (1mM) to activate PKA, and PMA (0.1μM) to activate PKC. Activation of PKA (Fig. 2, lane 3) but not PKC (Fig. 2, lane 6) was found to very significantly enhance the interaction of 14-3-3η with the α4 subunit. In the presence of the PKA inhibitor H-89 (30μM), the effect of forskolin was significantly attenuated (Fig. 2, lane 4) consistent
with the idea that PKA-dependent phosphorylation was involved in mediating the interaction.

**14-3-3η interacts with recombinant α4β2 AChRs expressed in tsA 201 cells**

To determine whether 14-3-3η could also interact with recombinant α4β2 AChRs in mammalian cells, we immunoisolated 1% NP-40-solubilized recombinant α4β2 AChRs using anti-α4 subunit mAb beads from tsA 201 cells transfected with the α4, β2, and 14-3-3η cDNAs. As a control for nonspecific binding, we used beads coupled to nonspecific rat IgG. We observed immunoreactivity for 14-3-3η and the α4 subunit migrating at their expected molecular masses of ~30kDa and ~70kDa, respectively (Fig. 3A, lane 2). No immunoreactivity for either protein in the control nonspecific rat IgG lane was observed (Fig. 3, lane 1).

As with the α4 subunit alone, we tested if activation of kinases would increase the interaction of 14-3-3η with recombinant α4β2 AChRs. We treated transfected cells coexpressing 14-3-3η and recombinant α4β2 AChRs with forskolin (50µM) plus IBMX (1mM) to activate PKA, and PMA (0.1µM) to activate PKC. Activation of PKA (Fig. 3A, lane 3) but not PKC (Fig. 3A, lane 6) was found to very significantly enhance the interaction of 14-3-3η with recombinant α4β2 AChRs. In the presence of the PKA inhibitor H-89 (30µM), the effect of forskolin was significantly attenuated (Fig. 3A, lane 4).
The preceding results were obtained by immunoisolating recombinant α4β2 AChRs with anti-α4 mAb beads. Since this mAb binds both assembled and unassembled α4 subunits, we were unable to distinguish if 14-3-3η interacted with α4 subunits that were unassembled, or assembled with the β2 subunits. To test if 14-3-3η could interact with assembled α4 subunits, α4β2 complexes were isolated with the anti-β2 mAb beads. The anti-β2 mAb used (mAb 295) binds the conformationally mature β2 subunit only. Reactivity with denatured β2 subunits on immunoblots is not observed. We found that activation of PKA by forskolin significantly enhanced the interaction of 14-3-3 with α4β2 AChR complexes immunoisolated with the anti-β2 mAb beads both in the absence (Fig. 3B, lane 2) and the presence of exogeneous 14-3-3η (Fig. 3B, lane 4). We also observed low basal levels of interaction of 14-3-3 with α4β2 AChR complexes even prior to activation of PKA by forskolin (Fig 3B, lanes 1 and 3). These results suggested that both endogeneous 14-3-3 and exogeneous 14-3-3η associated with the α4β2 AChR complexes. In addition, in three independent experiments we also observed that both in the presence or absence of exogeneous 14-3-3η, the amount of α4 subunits immunoprecipitated by the anti-β2 mAb beads from detergent extracts of cells treated with forskolin was consistently higher than that from detergent extracts of cells not treated with forskolin. The increased amounts of the α4 subunits also correlated with greater amounts of 14-3-3 coimmunoprecipitated with the α4β2 AChR complexes using the anti-β2 mAb beads suggesting a possible role for 14-3-3η in altering the steady state levels of α4β2 AChR complexes.
To further demonstrate that the interaction of 14-3-3\(\eta\) with the \(\alpha4\beta2\) AChRs was phosphorylation dependent, the detergent-solubilized \(\alpha4\beta2\) AChR immunoisolated complexes from cells treated with forskolin and IBMX, were treated with recombinant protein phosphatase I (PPI). We observed a significant reduction in the amount of 14-3-3\(\eta\) associated with immunopurified recombinant \(\alpha4\beta2\) AChRs treated with PPI compared to those treated with the buffer alone under identical conditions (Fig. 3C). These results further supported the fact that forskolin-dependent enhancement of the interaction of 14-3-3\(\eta\) with \(\alpha4\beta2\) AChRs was due to a PKA-mediated phosphorylation event.

14-3-3\(\eta\) fails to interact with mutant \(\alpha4^{S441A}\beta2\) AChRs

We examined if 14-3-3\(\eta\) could associate with mutant \(\alpha4^{S441A}\beta2\) AChRs, in which serine441 of the \(\alpha4\) subunit was mutated to alanine, following activation of PKA by forskolin in tsA201 cells. In keeping with the yeast two-hybrid mapping and mutagenesis studies, mutating serine 441 to alanine almost completely abolished interaction of 14-3-3\(\eta\) with mutant \(\alpha4^{S441A}\) subunit (Figure 2, lane 5) and the mutant \(\alpha4^{S441A}\beta2\) AChRs (Figure 3A, lanes 5; Figure 3B, lanes 5-8). These results, in conjunction with the results from previous studies of several other investigators demonstrating that a phosphoserine enhances the interaction of 14-3-3 with its target protein, suggested that serine 441 is the most likely target of PKA phosphorylation.
14-3-3η stabilized the wild-type α4 subunit but not the mutant α4\(^{S441A}\) subunit

Since 14-3-3η bound to the α4 subunit alone, we examined if 14-3-3η, a chaperone protein had a role in the early biogenesis of the α4 subunit. The α4 and the α4\(^{S441A}\) subunits were separately cotransfected with or without 14-3-3η cDNA into tsA201 cells. We studied the influence of the presence of 14-3-3η on the α4 or α4\(^{S441A}\) subunit steady state levels prior to, and following, activation of PKA by forskolin. To ensure differences were not simply due to variability in transfections between wells treated similarly, each condition was independently processed and analyzed in duplicate (indicated by a bar over the two lanes in Figure 4). Prior to activation of PKA, the presence of 14-3-3η did not significantly alter the steady state levels of the α4 subunit (lane 2 compared to lane 1) or that of the α4\(^{S441A}\) subunit (lane 6 compared to 5). In the absence of 14-3-3η, activation of PKA by forskolin did not significantly alter the steady state levels of the α4 subunit (lane 3 compared to lane 1) or the α4\(^{S441A}\) subunit (lane 7 compared to lane 5). However, a very significant increase (at least 5-fold) in the steady state levels of the wild-type α4 subunit was observed after treatment with forskolin in the presence of 14-3-3η (lane 4 compared to lane 2) in contrast to virtually no change in the steady state levels of the mutant α4\(^{S441A}\) subunit under similar conditions (lane 8 compared to lane 6). A similar result was obtained when cells were treated with forskolin (50µM) for only 1 hour instead of 24 hours (data not shown). These results indicated that a significant increase in the steady state levels of the α4 subunit occurred in the presence of 14-3-3η and following activation of PKA. These results, in conjunction with the
increase in steady state levels of α4β2 AChRs support a role for 14-3-3η in regulating the stability of the α4 subunit through a PKA-dependent phosphorylation mechanism.

Higher surface expression of wild-type α4β2 AChRs than mutant α4S441Aβ2 AChRs

To determine whether the interaction of 14-3-3η with the α4 subunits altered the cell surface expression levels of α4β2 AChRs, we monitored these levels for both wild-type α4β2 AChRs and mutant α4S441Aβ2 AChRs using an enzyme-linked immunoassay. Because 14-3-3 interacts with α4β2 AChRs following activation of PKA, we also examined if activation of PKA by forskolin (10µM) altered cell surface expression levels of wild-type α4β2 AChRs and mutant α4S441Aβ2 AChRs. The modified enzyme-linked immunoassay we used has been used previously to measure the surface expression of α4β2 AChRs (17). In our assay we measured the relative amount of the β2 subunit in cells treated under the described conditions with an anti-β2 subunit primary antibody. The amount of β2-immunoreactivity was then determined using an HRP-conjugated secondary Ab. The amount of secondary Ab bound to the primary mAb was then determined by measuring HRP enzymatic activity of the conjugated enzyme on a substrate (3, 3', 5, 5'-tetramethylbenzidine) whose product is colored blue, and whose concentration can then be determined spectrophotometrically. As controls for nonspecific binding of the Abs, we used cells transfected with the vector alone. The surface expression of wild-type α4β2 AChRs was found to be ~2-fold higher than the mutant α4S441Aβ2 AChRs (Figure 5). Following treatment with forskolin (10µM), the wild-type α4β2 AChRs showed a small but statistically significant increase (~20%, n=7,
p<0.005) in their cell surface expression levels. In contrast, forskolin did not induce a statistically significant change in the cell surface expression levels of mutant α4<sup>S441A</sup>β2 AChRs. The 2-fold difference between the surface expression levels of the α4β2 AChRs and the α4<sup>S441A</sup>β2 AChRs was observed with two different preparations of cDNAs making it very unlikely that it was due to differences in transfection efficiencies between the α4 subunit cDNA and the α4<sup>S441A</sup> subunit cDNA due to differences in the quality of the DNA samples. Similar results in the absence of transfected exogeneous 14-3-3η (data not shown) in keeping with our findings that the endogeneous 14-3-3 associated with α4β2 AChRs under these conditions.

Functional consequences of coexpressing 14-3-3η with wild-type α4β2 AChRs and mutant α4<sup>S441A</sup>β2 AChRs in Xenopus oocytes

We determined the functional consequences of the interaction of 14-3-3η with α4β2 AChRs or α4<sup>S441A</sup>β2 AChRs by studying their electrophysiological properties. AChR subunits were expressed from in vitro transcribed cRNAs microinjected into oocytes and currents elicited by 4 sec applications of different concentrations of ACh recorded using two-electrode voltage clamp methodology. ACh elicited dose-dependent response from both wild-type α4β2 AChRs and mutant α4<sup>S441A</sup>β2 AChRs when expressed alone or when coexpressed with 14-3-3η and treated with forskolin (50µM) for 4 h at room temperature. The whole cell currents are shown in Fig. 6 (top panel). Currents for both the wild-type α4β2 AChRs and mutant α4<sup>S441A</sup>β2 AChRs showed characteristic slow desensitization currents previously described for neuronal α4β2 AChRs. Both wild-type
α4β2 AChRs and mutant α4S441Aβ2 AChRs gave concentration/response curves that were best fit by a one-site Hill equation (Fig. 6; bottom panel). ACh activated the wild-type α4β2 AChR with an EC$_{50}$ = 41±3µM ($n_H$=1.5) and the wild-type α4β2 AChR coexpressed with 14-3-3η with an EC$_{50}$ = 64±2µM ($n_H$=2.0) after treatment with forskolin. ACh activated the mutant α4S441Aβ2 AChR with an EC$_{50}$ = 61±3µM ($n_H$=2.2) and the mutant α4S441Aβ2 AChR coexpressed with 14-3-3η with an EC$_{50}$ = 55±1µM ($n_H$=2.0) after treatment with forskolin. Because the whole cell current activated by ACh and the EC$_{50}$'s for AChR activation were not significantly different, these results suggested that 14-3-3η was unlikely to have a role in modulation of the functional properties of α4β2 AChRs.

**Immunohistochemical localization of 14-3-3 and the α4 subunit in transfected cells**

We compared the distribution of 14-3-3 proteins with that of the α4 subunit at the single cell level in transfected cells treated with and without forskolin (10µM). Transfected cells were fixed with methanol/acetone and then sequentially immunostained for the α4 subunit followed by staining for 14-3-3 as described in the experimental procedures section. Antibody binding was then visualized by confocal immunofluorescence microscopy using goat anti-mouse Alexa Fluor 546 conjugated Abs and the goat anti-rat Alexa Fluor 488 conjugated Abs. At the single cell level, diffuse immunostaining for the α4 subunit (red, top panel, Fig 7) was observed throughout the ER/Golgi compartments and the surface membrane. In contrast staining for 14-3-3 was very distinctively different and was confined to the cytosolic region (green, top panel, Fig. 7). Some colocalization was evident in the merged images in the cytosolic region.
(yellow, top panel, Fig. 7). Following treatment with forskolin, colocalization within the ER/Golgi compartments was significantly enhanced, but no significant colocalization was evident at the surface membrane (yellow, bottom panel, Fig 7). As controls, we immunostained nontransfected cells and observed only very weak and diffuse staining with the 14-3-3 Ab to endogeneous 14-3-3 proteins and no detectable nonspecific staining with the α4 mAb (not shown). Thus, the colocalization results complemented the coimmunoisolation results and suggested that 14-3-3η interacted with the α4 subunit and α4β2 AChRs primarily within the ER/Golgi compartments of cells. It also complemented the results of our functional studies by showing that the reason why no significant change in the functional properties of α4β2 AChRs was observed when they were coexpresssed with 14-3-3η was most likely because 14-3-3η did not colocalize with surface α4β2 AChRs.

**Interaction of 14-3-3 with native α4β2 AChRs from rat brain**

To validate the physiological importance of the interaction of 14-3-3η with the α4 subunit in yeast, and with recombinant α4β2 AChRs in transfected cells, we determined if 14-3-3 is associated with native α4β2 AChRs immunopurified from rat brain. Rat brain membranes were solubilized using 1% NP-40 and the α4β2 AChRs immunopurified using anti-α4 subunit-specific mAbs and anti-β2 subunit-specific mAbs. Detergent-solubilized brain membrane extracts were also incubated with beads coupled to a control Ab (rat IgG). The interaction of 14-3-3 with α4β2 AChRs was then detected by immunoblotting with an anti-14-3-3 mAb. 14-3-3 was found to be associated with complexes of native
NP-40-solubilized α4β2 AChRs (Figure 8). The significant 14-3-3 immunoreactivity detected with α4β2 AChRs immunopurified with two different mAbs to the α4β2 AChRs compared to the absence of any detectable immunoreactivity among proteins that bind nonspecifically to the control Ab, suggested that the association with the complex was specific. This result also strongly supported the idea that the interaction of 14-3-3 with α4β2 AChR is physiologically significant *in vivo.*
DISCUSSION

The cloning of a multitude of neuronal AChR subunit cDNAs has revealed a great diversity of AChR subtypes whose functions in the nervous system remain enigmatic (19). The large cytoplasmic domain between the third and fourth transmembrane domain is highly divergent among the subunits (20). Some aspects of the roles subserved by the large cytoplasmic domain such as the polarized trafficking of AChR in neurons (21), the clustering of muscle AChRs at synaptic membrane subsites (22-24) are known.

Identification of proteins that interact with the cytoplasmic domain is likely to provide a better understanding of proteins involved in the subunit assembly, trafficking, clustering, and functions of AChRs. As a first step toward understanding which proteins interact with the widely expressed neuronal α4 AChRs, we used the α4 subunit cytoplasmic domain in a yeast two-hybrid screen. In this paper, we describe the identification of the first protein known to interact with the α4 subunit, 14-3-3η, and the characterization of its interaction with recombinant and native α4β2 AChRs. The results of our study provide novel mechanistic insights into the cellular events that mediate the interaction of 14-3-3η with the AChR α4 subunit following activation of PKA, and the consequences of this interaction on the stability of the subunit.

The seven member family of 14-3-3 proteins are intracellular proteins known to have a regulatory role in diverse functions through the activation, inhibition, and structural stabilization of numerous proteins (14,25). 14-3-3 is known to bind the
proteins BAD (26), apoptosis signal regulating kinase-1 (ASK1) (27) and α-synuclein (28). Interestingly, α-synuclein, a protein implicated in the neuropathology associated with some inherited forms of Parkinson's disease (29), shares some sequence homology with 14-3-3 and heterodimerizes with it (28). 14-3-3 proteins have also been implicated in modulating regulated exocytosis of neurotransmitters at presynaptic terminals (30-32).

14-3-3 proteins have previously been shown to bind to the sequence motif (RSxSx(P) where x = any amino acid), in which, phosphorylation of the second serine residue (underlined) is critical for the binding of 14-3-3 (33). The arginine residue at the first position appears to be essential but other residues are tolerated at the position of the proline residue. The yeast two-hybrid mapping studies using nested deletions of the α4 cytoplasmic domain allowed us to localize a potential 14-3-3 binding site (RSLS441VQ) between residues 413 and 450. Using site-directed mutagenesis we show that changing serine 441 to alanine nearly completely abolished the interaction of 14-3-3η with the mutated α4 bait. Interestingly, a second motif (RSRSIQ) closely resembling the consensus 14-3-3 binding-site motif is also present in the α4 subunit between residues 459 and 464 but was not essential for interaction with 14-3-3η.

We have provided several lines of evidence that suggests that serine 441 is phosphorylated by PKA. By treating transfected tsA201 cells with either forskolin to activate PKA, or PMA to activate PKC, we demonstrated that 14-3-3η binding to the recombinant α4 subunit is most robust following activation of PKA and not PKC and this effect is attenuated by the PKA blocker H-89. We also showed that there was a very
significant reduction in the amount of 14-3-3 associated with the $\alpha 4\beta 2$ AChR complex following treatment with the protein phosphatase PPI. Furthermore, 14-3-3$\eta$ fails to interact with recombinant $\alpha 4^{S441A}$ subunits alone, or $\alpha 4^{S441A}\beta 2$ AChRs, following treatment of cells with forskolin. In addition, serine 441 of the $\alpha 4$ subunit is within a predicted PKA phosphorylation site (as determined by sequence analysis using the phosphobase program from the CMS Molecular Biology Resource at www.unl.edu/stc-95/ResTool/cmshp.html) and numerous previous studies have indicated that the binding of 14-3-3 to most of their other target proteins is mediated by a phosphoserine or phosphothreonine residue (15,34).

We observed that activation of PKA significantly enhanced the interaction of 14-3-3$\eta$ with unassembled $\alpha 4$ subunits and with assembled $\alpha 4\beta 2$ AChR complexes. We have however failed to detect an increase in association of 14-3-3$\eta$ with $\alpha 4\beta 2$ AChRs in tsA201 cells following acute or chronic (24h) exposure of AChRs to nicotine (data not shown). These results suggest that other intracellular processes, other than channel activity, possibly governs the interaction of 14-3-3 with the $\alpha 4$ subunit and $\alpha 4\beta 2$ AChRs.

We have provided compelling evidence for a role of 14-3-3$\eta$ in increasing the stability of the $\alpha 4$ subunit and $\alpha 4\beta 2$ AChR under conditions that also correlate well with those that favor interaction of 14-3-3 with the $\alpha 4$ subunit. When $\alpha 4$ subunits are expressed alone, the wild-type $\alpha 4$ and mutant $\alpha 4^{S441A}$ subunits did not show significant differences in their steady state levels. However, we observed a very significant increase in the steady state levels of only the wild-type $\alpha 4$ subunit and not the mutant $\alpha 4^{S441A}$
subunit following activation of PKA by forskolin only in the presence of 14-3-3\(\eta\). Corresponding differences in the steady state levels of the \(\alpha_4\beta_2\) AChR and the \(\alpha_4^{S441A}\beta_2\) AChRs were also observed and strongly suggested that 14-3-3\(\eta\) plays a role in early posttranslational events that govern subunit and \(\alpha_4\beta_2\) AChR stability.

The phosphorylation of the \(\alpha_4\) subunit at serine 441 by PKA and its subsequent interaction with 14-3-3 alters cell surface \(\alpha_4\beta_2\) AChRs by increasing the \(\alpha_4\) subunit and \(\alpha_4\beta_2\) AChR steady state levels. In keeping with such a role for 14-3-3, we observed a correlation between higher cell surface expression levels of wild-type \(\alpha_4\beta_2\) AChRs and its ability to bind 14-3-3\(\eta\) and lower surface expression levels of the mutant \(\alpha_4^{S441A}\beta_2\) AChRs and their inability to bind 14-3-3\(\eta\). Furthermore we observed a small but significant increase in their cell surface expression levels following treatment with forskolin. In contrast, forskolin did not induce a significant change in the cell surface expression levels of mutant \(\alpha_4^{S441A}\beta_2\) AChRs. Similar results in surface expression levels following treatment with forskolin were observed in the absence of exogenous 14-3-3\(\eta\) and were most probably due to the observed ability of endogeneous 14-3-3 proteins to interact with \(\alpha_4\beta_2\) AChRs.

Previously, it has been reported that activation of PKA by forskolin results in a ~200% increase in cell surface expression of recombinant human \(\alpha_4\beta_2\) AChRs expressed in tsA201 cells (35). However, we do not observe such a large increase in surface expression of rat \(\alpha_4\beta_2\) AChRs expressed in tsA201 cells. We suggest that this difference perhaps reflects differences in the growth conditions and species-specific differences
(human versus rat) that might also affect the intrinsic efficiency of subunit assembly. The rather small but statistically significant increase (~20%) in surface expression levels of the wild-type α4β2 AChRs following treatment with forskolin is consistent with the idea that when subunit assembly was efficient, PKA-dependent phosphorylation only marginally contributes to further increases in surface expression.

The role of phosphorylation in regulating subunit assembly and cell surface expression is better characterized for muscle-type AChRs (36-43). In muscle-type AChRs, pulse chase experiments and immunofluorescent microscopy indicate that AChR subunit assembly is complete in the ER following which AChR oligomers move rapidly through the Golgi membrane onto the plasma membrane (37). Interestingly it has been demonstrated that both the γ and δ subunits are phosphorylated in vivo, and the δ subunits is more highly phosphorylated in the unassembled than in the assembled state indicating that phosphorylation precedes assembly and that phosphorylation/dephosphorylation mechanisms control AChR subunit (36). Furthermore, using Torpedo AChR subunits expressed in mouse fibroblasts, it has been previously demonstrated that cAMP-induced increase in expression of cell surface AChRs is due to phosphorylation of the unassembled γ subunit assembly (37). But the underlying mechanism by which this phosphorylation increases the efficiency of subunit assembly and increased surface AChR expression, has not been elucidated.

We have demonstrated that phosphorylation of the unassembled α4 subunit and the subsequent association of 14-3-3 with it increases it steady state levels in nonneuronal
cells. This mechanism is consistent with such a proposed role for 14-3-3 in regulating the turnover of the plasma membrane H⁺-ATPase (44). In addition, both PKA (45) and 14-3-3 isoforms (46) have been previously demonstrated to be appropriately localized to the ER/Golgi compartments to participate in such a process. Our results do not identify which exact isoform(s) of 14-3-3 is associated with the native α4 AChR subunit because the anti-14-3-3 mAb we used cross-reacts with several members of the 14-3-3 family. The family of 14-3-3 proteins consists of closely related members that do not show measurable differences in their affinities for a consensus binding site motif in vitro (33), though their binding in vivo is regulated by modulating their expression levels (47-49) and by phosphorylation of the 14-3-3 proteins themselves (50-52). Thus, the identity of the particular isoform(s) of 14-3-3 that binds the native α4 AChR subunit in vivo, remains to be determined. Because 14-3-3 can dimerize and thus simultaneously bind two different proteins, further experimentation will be needed to establish if other proteins are also involved in this process.

Finally, we would like to point out a possible pathophysiological significance of our work. It is well established that chronic intake of nicotine in smokers increases the expression levels of α4β2 AChRs in their brains (53). Because schizophrenics are very heavy smokers, a recent study showing reduced levels of [³H] nicotine binding sites, mostly to α4β2 AChRs (5), in the brains of schizophrenics compared to those of normal smokers (54) suggests that schizophrenics could have a possible defect in processes that regulate cell surface expression levels of their α4β2 AChRs. However, it is likely that schizophrenics have deficits that are not limited to reduced levels of α4β2 AChRs.
Interestingly, recent genetic analyses of allelic frequencies of a variable number of tandem repeat in the 5'-noncoding region of the 14-3-3\textit{\textgreek{n}} gene suggests that it is a potential susceptibility gene for schizophrenia, particularly for early-onset schizophrenia (55). It has been previously reported that the 14-3-3\textit{\textgreek{n}} gene has a CRE binding site in its promoter (56) and as such its expression levels is likely to be regulated by changes in cellular levels of cAMP through the activation of the transcription factor CREB. Thus we speculate that 14-3-3\textit{\textgreek{n}} could have a broader role in regulating the excitability of neurons in an activity-dependent manner by modulating the levels of other proteins necessary for adaptive changes within specific neural networks. An understanding of the physiological significance of the interaction of 14-3-3 with native AChR \textit{\textalpha}4 subunits might be better understood by modulation of the expression levels of 14-3-3\textit{\textgreek{n}} \textit{in vivo}. 
14-3-3η Stabilizes Nicotinic Receptor α4 Subunits

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FIGURE LEGENDS

Fig. 1. Mapping interaction of 14-3-3η within the α4 cytoplasmic domain.

A) Residues 413-450 of the α4 cytoplasmic domain mediate interaction with 14-3-3η. Six nested C-terminal deletions of the α4 cytoplasmic domain were tested for their ability to interact with 14-3-3η protein in the yeast two-hybrid system. Positive clones were determined by the ability of yeast cells to grow on plates lacking leucine, tryptophan, histidine, and for their ability to turn blue on media supplemented with X-gal. Plates lacking histidine and tryptophan (his trp) select for the presence the plasmids carrying the bait and the library protein. Plates lacking histidine, tryptophan, and leucine (his trp leu) additionally select for interaction between the bait protein and the interaction protein.

B) Serine 441 is essential for interaction of 14-3-3η with the cytoplasmic domain of the α4 AChR subunit. Interaction of 14-3-3η the wild-type α4 bait versus a mutant α4 bait in which residue serine 441 (underlined) is mutated to alanine within a putative 14-3-3 consensus binding-site motif RSLΔVQ.

Fig. 2. Forskolin treatment increases 14-3-3η binding to α4 subunits expressed alone. Cells were treated with (+) or without (-) forskolin (50μM) plus IBMX (1mM) with or without the PKA inhibitor (H-89; 30μM); or PMA (0.1μM). Proteins were solubilized with 1%NP-40 from cells transfected with α4 and 14-3-3η cDNAs and immunopurified (IP) with anti-α4 subunit mAb beads.
Fig. 3. Interaction of 14-3-3η with recombinant α4β2 AChRs.

A) Cells were transfected with the α4, or α4^{S441A}, β2 and 14-3-3η cDNAs. Cells were treated with forskolin (50μM) plus the cAMP phosphodiesterase inhibitor IBMX (1mM) (lanes 3, 4, and 5) after 2 h incubation of cells with (lane 4) or without the PKA inhibitor (H-89; 30μM) (lane 3). Cells were treated with PMA (0.1μM) (lane 6). After treatment, proteins were solubilized with 1%NP-40 and proteins immunopurified (IP) with beads coupled to anti-α4 subunit mAbs and with beads coupled to nonspecific rat IgG Abs (lane 1). Immunopurified proteins were fractionated by SDS-PAGE and the top half of the blots immunoblotted (IB) with the anti-α4 subunit mAb and the bottom half with the anti-14-3-3 mAb.

B) In a separate experiment, cells were transfected with the α4, β2 cDNAs and with or without the 14-3-3η cDNA. Cells were treated with or without forskolin (10μM), and the immunocomplexes isolated with anti-β2 subunit mAb beads. The final immunoblot analysis of all samples eluted from the beads was done the same way as described in 2A.

C) An aliquot of the immunocomplexes tethered to the anti-β2 mAb beads was separated into two pools, one of which was treated with (+) and the other without (-) protein phosphatase 1 (PPI) for 1 hour at 30°C. The final immunoblot analysis of all samples eluted from the beads was done the same way as described in 2A.
**Fig. 4.** 14-3-3η stabilizes the α4 wild-type subunit not the mutant α4 S441A subunit. Cells were cotransfected with the α4 cDNA (lanes 1-4) or α4 S441A cDNA (lanes 5-8), with 14-3-3η cDNA (lanes 2, 4, 6, 8) or without 14-3-3η cDNA (vector pEF6A alone) (lanes 1, 3, 5, 7). The following day, the cells were treated with or without forskolin (10µM). The bar across the paired lanes represent samples treated under identical conditions from independently transfected and processed samples from a single experiment. Forty eight hours after transfection, the cells were lysed in 500µl of 2% NP-40 lysis buffer and the proteins solubilized for 3 hours. The lysates were centrifuged and 20µl of supernatant were fractionated by SDS-PAGE. The top half of the blots were immunoblotted with the anti-α4 subunit mAb and the bottom half with the anti-14-3-3 mAb.

**Fig. 5.** Higher surface expression of wild-type α4β2 AChRs than mutant α4S441Aβ2 AChRs.

The surface expression levels of wild-type α4β2 AChRs and mutant α4S441Aβ2 AChRs was determined following treatment of cells with forskolin (10µM, 24 h). The relative amount of primary anti-β2 subunit mAb bound to the surface AChRs was quantitated using a HRP-conjugated secondary Ab in conjunction with the HRP substrate (3, 3', 5, 5'-tetramethylbenzidine) in a colorimetric assay as described in the "Experimental Procedures" section. The bar graphs represent the normalized levels of AChR after substraction of the mean background value obtained from cells transfected with the vector alone. Each experiment was done in duplicate. The error bars represent the S.E. of measurements from seven separate experiments.
Fig. 6. Functional properties of $\alpha_4\beta_2$ AChRs and $\alpha_4^{S441A}\beta_2$ AChRs coexpresssed with 14-3-3$\eta$.

**Top: Currents induced by ACh.** Currents induced by 4 sec applications of different concentrations of ACh are shown for *Xenopus* oocytes expressing $\alpha_4\beta_2$ AChRs, $\alpha_4\beta_2$ AChRs + 14-3-3$\eta$ (treated with forskolin), $\alpha_4^{S441A}\beta_2$ AChRs and $\alpha_4^{S441A}\beta_2$ AChRs + 14-3-3$\eta$ (treated with forskolin). The oocytes were clamped at a holding potential of -70mV. ACh was applied successively following 4 min wash out periods following each application of ACh.

**Bottom: Concentration/response curves of ACh.** Data obtained from 2-3 oocytes held at -70mV were normalized to the control response induced by 1mM ACh, averaged, and fit using the Hill equation. The error bars represent the S. E.

Fig. 7. Colocalization of 14-3-3 and AChR $\alpha$4 subunit in transfected tsA 201 cells.

Transfected cells were fixed and processed for immunohistochemistry. Immunofluorescence was detected by confocal microscopy as described in the Experimental Procedures. **Top panel:** transfected with $\alpha_4 + \beta_2 + 14$-3-3$\eta$ cDNAs. **Bottom panel:** transfected with $\alpha_4 + \beta_2 + 14$-3-3$\eta$ cDNAs, and treated with forskolin (10$\mu$M). The images are 1$\mu$m thick optical sections through single cells in culture. $\alpha_4$ immunoreactivity was visualized by the binding of Alexa Fluor 488-conjugated secondary Abs (red) and 14-3-3 immunoactivity was visualized by the binding of Alexa Fluor 546-conjugated secondary Abs (green). Superimposition of the two images is shown in the third panel (yellow). Original magnification of all images was 600X.
Fig. 8. 14-3-3 coimmunopurifies with native detergent-solubilized α4β2 AChRs.

1%NP-40 detergent-solubilized α4β2 AChRs were immunopurified (IP) from rat brain membrane. Proteins eluted from specific mAb beads (mAb 299 to the α4 subunit; mAb 295 to the β2 subunit) and control mAb beads (rat IgG) were fractionated by SDS-PAGE and immunoblotted (IB) with the anti-14-3-3 mAb, the anti-α4 mAb, and the anti-β2 antiserum. The protein lysate represents ~1/5,000 of the total solubilized protein used in each of the IP.
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### Fig. 1

**A**

| 302 | α4 Cytoplasmic Domain Baits | 561 | His-Trp- | His-Trp-Leu-X-gal |
|-----|-----------------------------|-----|---------|--------------------|
|     |                             |     | +       | +                  |
|     |                             |     | +       | +                  |
|     |                             |     | -       | -                  |
| 376 |                             |     | +       | +                  |
| 339 |                             |     | -       | -                  |

**B**

| 302 | Wild-type α4 Bait | 561 | His-Trp- | His-Trp-Leu-X-gal |
|-----|-------------------|-----|---------|--------------------|
|     |                   |     | +       | +                  |

![](image)

| Mutant α4 Bait | 441 |
|----------------|-----|
| RSLSVQ         |     |
| RSLAVQ         |     |

![](image)
| Transfected Subunit | IgG | α4 mAb |
|---------------------|-----|--------|
|                     | α4  | α4     | α4     | α4     | α4S441A | α4     |

**IB: α4**

- 96 kDa

**IB: 14-3-3**

- 35.4 kDa

**Forsk**
- - - + + + + -

**H-89**
- - - - + - - -

**PMA**
- - - - - - - +

**Fig. 2**
| IP:         | IgG       | α4 mAb     |
|-------------|-----------|------------|
| **AChR**    | α4β2      | α4β2       | α4β2       | α4β2       | α4S441Aβ2   | α4β2       |          |
| **IB:α4**   |           |            |            |            |             |            | - 96     |
| **IB:14-3-3** |          |            |            |            |             |            | - 35.4   |

| forsk       | -         | -          | +          | +          | +           | +          | -         |
| H-89        | -         | -          | -          | +          | -           | -          | -         |
| PMA         | -         | -          | -          | -          | -           | -          | +         |

Fig. 3A
| AChR Subunit | α4β2 | α4S441Aβ2 |
|--------------|------|----------|
| IB: α4       | ![Alpha4 Image] | ![Alpha4 Image] |
| IB: 14-3-3   | ![14-3-3 Image] | ![14-3-3 Image] |

Forsk: - - + + - + - + +
14-3-3η: - - + + - - + + +

Fig. 3B
| IP: AChR | β2 mAb | α4β2 | α4β2 | kDa |
|----------|-------|------|------|-----|
| IB: α4   |       |      |      | 96  |
| IB: 14-3-3|      |      |      | 35.4|
| Forsk    | +     | +    |      |     |
| PPI      | -     |      | +    |     |

Fig. 3C
| AChR Subunit | α4  | α4S441A |
|--------------|-----|---------|
| 1            |     |         |
| 2            |     |         |
| 3            |     |         |
| 4            |     |         |
| 5            |     |         |
| 6            |     |         |
| 7            |     |         |
| 8            |     |         |

**IB: α4**

| KDa | -208 | -131 | -96 |

**IB: 14-3-3**

| KDa | -354 |

**Forsk**

- - + + - - + + + +

**14-3-3η**

- + - + - + - + +
Fig. 5

**α4β2 AChRs + 14-3-3η**

**α4S441Aβ2 AChRs + 14-3-3η**

- **p<0.005; **
- **p>0.05; NS**
| IP:     | IgG | α4   | β2   | Lysate | kDa |
|---------|-----|------|------|--------|-----|
| IB: α4  |     | ![Image](image1) | ![Image](image2) | ![Image](image3) | -79 |
| IB: β2  | ![Image](image4) | ![Image](image5) | ![Image](image6) | ![Image](image7) | -43.9 |
| IB: 14-3-3 | ![Image](image8) | ![Image](image9) | ![Image](image10) | ![Image](image11) | -29.3 |

**Fig. 8**
The chaperone protein 14-3-3 interacts with the nicotinic acetylcholine receptor α4 subunit: Evidence for a dynamic role in subunit stabilization

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