The Chaperone Protein 14-3-3η Interacts with the Nicotinic Acetylcholine Receptor α4 Subunit

EVIDENCE FOR A DYNAMIC ROLE IN SUBUNIT STABILIZATION*

By using the large cytoplasmic domain of the nicotinic acetylcholine receptor (AChR) α4 subunit as a bait in the yeast two-hybrid system, we isolated the first cytosolic protein, 14-3-3η, known to interact directly 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 by forskolin. The interaction of 14-3-3η with recombinant α4 subunits was abolished when serine 441 of the α4 subunit was 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 EC_{50} values for activation by acetylcholine 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.

Neuronal nicotinic acetylcholine receptors (AChR) 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 are assembled from different combinations of α2–α9 and β2–β4 subunits have been identified (3, 4). Of these, the α4β2 AChR is widely expressed in the central nervous system and represents >80% of the high affinity [3H]nicotine-binding sites in mammalian brain (5). Our understanding of their physiological roles comes most recently from gene knock-out studies in mice. In which the α4 subunit gene has been deleted lack [3H]nicotine- or [3H]epibatidine-binding sites in their brain and exhibit reduced anticonvulsive effects of nicotine (6). Mice in which the β2 subunit gene has been deleted also show little [3H]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 α4β2 AChRs have a role in mediating addiction to nicotine. The normal and pathophysiological functions mediated by α4β2 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 α4β2 AChRs harboring at least two separate mutations within their α4 subunit (9–12). Most recently, α4β2 AChRs, among other β2 subunit-containing AChRs, have been implicated in neuronal survival during aging, 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 interact directly 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, ζ, ε, ι, and 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 PKA. 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.) and Pfu Turbo (Stratagene, Inc., La Jolla, CA). All DNA sequence analysis was done using the ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech). For all primers, the restriction enzyme sites are underlined. 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 TCA GAG GGT GCC TCG CGG CCG CCG-3′ and the reverse primer 5′-GCC TTC TCA TCA TGG GTG CAC GAG AAG GAA GCT-3′. The full-length cDNAs were ligated into the pET6/myc-His A (Invitrogen, Carlsbad, CA).

Yeast Two-hybrid Library Screen—Yeast two-hybrid screens were performed with yeast a4 bait plasmid pLexA and the p8op-LacZ bait plasmid pLexA, both of which carry a single copy of the AChR α4 subunit (299) and anti-AChR 4S441A was generated by mutagenesis using the following two primers: 5′-ATT CGG GCC CCG GG-3′ and 5′-GGG GGA TCC TCA GGT GCC TCC CGC CTT GAG-3′. The amplified cDNAs were ligated into the EcoRI-BamHI HI 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 TTC GAC CAG GAA GGT GGG-3′ and the reverse primer 5′-GGG TCT AGA TCA GTT GCC TTC TCC TGC TTC TTC-3′. The amplified cDNAs were ligated into the EcoRI-BamHI HI sites of the vector pLexA. The mouse 14-3-3 α subunit was expressed as a fusion protein with the plasmic domain (amino acids 302–561) of the rat α4 subunit in the ER/Golgi compartment (17). For all experiments, cells were used between passages 30 and 40. The experiments were performed with cells incubated at 30 °C following transfection.

Expression and Analysis of the α4 Subunit—To study the effect of 14-3-3 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 14-3-3 and incubated at 30 °C (the DNA concentrations and ratios were kept constant by using the pREP6 vector DNA). The transfected cells were treated 24 h with or without forskolin (10 μM). After washing once with ice-cold PBS, the cells were solubilized in 500 μl of the following lysis buffer: 50 mM NaCl, 30 mM trithionalamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 1 mM benzamidine, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 2% Nonidet P-40. After shaking for 3 h at 4 °C, the lysates were centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was then analyzed by SDS-PAGE.

Immunosolation of Recombinant AChRs in tsA201 Cells—tsA201 cells were washed twice with ice-cold PBS containing 50 mM NaF and 1 mM sodium orthovanadate lysed in 1 ml of lysis buffer (50 mM NaCl, 30 mM trithionalamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 mM sodium vanadate, 10 μM p-nitrophenyl phosphate, 25 μg/ml apronitin, 25 μg/ml leupeptin, 25 μg/ml pepstatin, 0.3 μM okadaic acid, 1 mM sodium tetrathionate, 1 μM N-ethylmaleimide, 50 μM phenylarsine oxide, and 1% Nonidet P-40), and agitated vigorously for 2 h at 4 °C. After centrifugation at 18,000 × g for 15 min, the clear supernatant from each sample (−1 ml) was incubated with 10 μl of mAb-coupled beads (that were preblocked with 5% non-fat milk for 30 min) for 24 h. The beads were then washed 5 times with 800 μl of solubilization buffer and eluted with sample buffer (lacking β-mercaptoethanol to avoid reduction of the disulfide 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.

Immunosolation of Native AChRs from Rat Brain—Frozen rat brain homogenates were obtained from the rat brain (250 mls), mAb (1 μl), and 30 mM NaCl, 30 mM trithionalamine, pH 7.5, 5 mM EGTA, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 2 mM sodium vanadate, 10 μM p-nitrophenyl phosphate, and 1% Nonidet P-40. To study the effect of the homogenation buffer containing 1% Nonidet P-40 and 25 μg/ml apronitin, 25 μg/ml leupeptin, 25 μg/ml pepstatin, 0.1 mM okadaic acid, 1 mM sodium tetrathionate, 1 mM N-ethylmaleimide, 50 μM phenylarsine oxide) for 2 h at 4 °C. The clear supernatant obtained after centrifugation of the pellet at 18,000 × g for 30 min was used for all subsequent immunosolation procedures. Detergent-solubilized brain extracts (typically 10 ml) thus obtained were incubated with−25 μl of mAb-coupled Actigel ALD beads (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 bound 14-3-3 proteins that are abundant in brain extracts and many of whose isoforms cross-react 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 disulfide 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 electrophoresed onto polyvinylidene difluoride membrane (IMMUN-BLOT; Bio-Rad), and the membranes were 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 was 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 described previously (17). Briefly, 48 h after transfection, ta2A01 cells plated in 12-well plates (0.5 × 10⁶ cells/well) were washed once in PBS and then blocked with PBS containing 3% BSA, and the cells were incubated for 1 h with an anti-β2 subunit mAb (295) in PBS containing 3% BSA at room temperature. After 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 300 μl of the HRP substrate 3,3′,5,5′-tetramethylbenzidine (Sigma) for 1 h. The absorbance of the supernatant was then measured at 655 nm in a Beckman 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 described previously (18). Oocytes were injected with 20 ng of cRNAs for the α4 and β2 subunits and 40 ng of 14-3-3θ per oocyte and incubated for 3–7 days at 16–18 °C in 50% L-15 medium (Life Technologies, Inc.) containing 10 mM HEPES buffer, pH 7.5.

**Electrophysiological Recordings—**Currents were measured using standard two-microelectrode voltage-clamp amplifier (Oocyte Clamp OC-725C) as described previously (18). Electrodes were filled with 3 M KCl and had resistances of 1.0–2.0 MΩ for the voltage electrode and 0.5–1.0 MΩ for the current electrode. All records were digitized at 200 Hz with MacLab software and hardware (AD Instruments).

**Immunohistochemistry—**Cells were fixed with an ice-cold mixture of 50% methanol, 50% acetone for 5 min, washed three times with 2 ml 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) fluorescein-conjugated anti-rat secondary Ab in PBS containing 4% NGS for 1 h at room temperature. The cells were then washed 3 times for 15-min periods in PBS 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 (catalog number A-11008) and the goat anti-mouse Alexa Fluor 546-conjugated Ab (catalog number A-11036) 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 fluorescein isothiocyanate and rhodamine filters. The resulting confocal images were captured on Silicon Graphics workstations. Images were pseudocolored and processed using Adobe Photoshop software.

**Fig. 1.** Mapping interaction of 14-3-3θ within the α4 cytoplasmic domain. A, residues 413–450 of the α4 cytoplasmic domain mediate interaction with 14-3-3θ. Six nested C-terminal deletions of the α4 cytoplasmic domain were tested for their ability to interact with 14-3-3θ protein in the yeast two-hybrid system. Positive clones were determined by the ability of yeast cells to grow on plates lacking leucine, tryptophan, and histidine and for their ability to turn blue on media supplemented with X-gal. Media lacking histidine and tryptophan (his’ trp’) select for the presence of the plasmids carrying the bait and the library protein. Media 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 14-3-3θ with the cytoplasmic domain of the α4 AChR subunit. 14-3-3θ interacts with the wild-type α4 bait but not a mutant α4 bait in which residue serine 441 (underlined) is mutated to alanine within a putative 14-3-3θ consensus binding-site motif RSLSVQ.

**Fig. 2.** Forskolin treatment increases 14-3-3θ binding to α4 subunits expressed alone. Cells were treated with (+) or without (−) forskolin (Forsk, 50 μM) plus IBMX (1 μM) with or without the PKA inhibitor (H-89, 30 μM) or PMA (0.1 μM). Proteins were solubilized with 1% Nonidet P-40 from cells transfected with α4 and 14-3-3θ cDNAs and immunopurified (IP) with anti-α4 subunit mAb beads. IB, immunoblot.
RESULTS

14-3-3γ 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 ~2 × 10^6 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 14-3-3γ that interacted with the α4 bait were obtained. A full-length clone of 14-3-3γ was chosen for further characterization.

To delineate the site where 14-3-3γ 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 14-3-3γ 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 14-3-3γ to residues 413–450 (Fig. 1A). A putative motif, RSLSVQ, in which the underlined serine residue is phosphorylated, has been demonstrated previously to be important for the binding of 14-3-3 proteins to some of its target proteins. A sequence that closely resembles this motif, RSILSVQ, 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 14-3-3γ with the α4 cytoplasmic domain bait in the yeast two-hybrid system (Fig. 1B). These results suggested that the high affinity binding of 14-3-3γ to the α4 cytoplasmic domain is mediated by serine 441 within a specific consensus binding site motif.

14-3-3γ Interacts with Recombinant α4 Subunits in tsA 201 Cells—To test directly if 14-3-3γ could interact with full-length unassembled α4 subunits alone, we transfected tsA 201 cells with the α4 subunit cDNA. Because it has been demonstrated previously 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 14-3-3γ with the recombinant α4 subunit. We treated transfected cells coexpressing 14-3-3γ and recombinant α4 subunits with forskolin (50 μM) plus IBMX (1 mM) to activate PKA and PMA (0.1 μM) to activate PKC. Activation of PKA (Fig. 2, 3rd lane) but not PKC (Fig. 2, 6th lane) was found to significantly enhance the interaction of 14-3-3γ with the α4 subunit. In the presence of the PKA inhibitor H-89 (30 μM), the effect of forskolin was significantly attenuated (Fig. 2, 4th lane).

Immunopurified proteins were fractionated by SDS-PAGE, and the top half of the blots were immunoblotted (IB) with the anti-α4 subunit mAb and the bottom half with the anti-14-3-3γ mAb. In a separate experiment, cells were transfected with the α4, β2 cDNAs and with or without the 14-3-3γ cDNA. Cells were treated with or without forskolin (10 μM), and the immunocomplexes were 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 Fig. 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 h at 30 °C. The final immunoblot analysis of all samples eluted from the beads was done the same way as described in Fig. 2A.

Fig. 3. Interaction of 14-3-3γ with recombinant α4β2 AChRs. A, cells were transfected with the α4 or α4Δ441, β2, and 14-3-3γ cDNAs. Cells were treated with forskolin (Forsk, 50 μM) plus the cAMP phosphodiesterase inhibitor IBMX (1 mM) (3rd to 5th lanes) after a 2-h incubation of cells with (4th lane) or without the PKA inhibitor (H-89; 30 μM) (3rd lane). Cells were treated with PMA (0.1 μM) (6th lane). After treatment, proteins were solubilized with 1% Nonidet P-40, and proteins were immunopurified (IP) with beads coupled to anti-α4 subunit mAbs and with beads coupled to nonspecific rat IgG Abs (1st lane).
consistent with the idea that PKA-dependent phosphorylation was involved in mediating the interaction.

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

As with the α4 subunit alone, we tested if activation of kinases would increase the interaction of 14-3-3-η with recombinant α4β2 AChRs. We treated transfected cells coexpressing 14-3-3-η and recombinant α4β2 AChRs with forskolin (50 μM) plus IBMX (1 mM) to activate PKA and PMA (0.1 μM) to activate PKC. Activation of PKA (Fig. 3A, 3rd lane) but not PKC (Fig. 3A, 4th lane) was found to very significantly enhance the interaction of 14-3-3-η with recombinant α4β2 AChRs. In the presence of the PKA inhibitor H-89 (30 μM), the effect of forskolin was significantly attenuated (Fig. 3A, 4th lane).

The preceding results were obtained by immunosolubilating recombinant α4β2 AChRs with anti-α4 mAb beads. Since this mAb binds both assembled and unassembled α4 subunits, we were unable to distinguish if 14-3-3-η interacted with α4 subunits that were unassembled or assembled with the β2 subunits. To test if 14-3-3-η 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-η with α4β2 AChR complexes immunosolubilated with the anti-β2 mAb beads both in the absence (Fig. 3B, 2nd lane) and the presence of exogenous 14-3-3-η (Fig. 3B, 4th lane). We also observed low basal levels of interaction of 14-3-3-η with α4β2 AChR complexes even prior to activation of PKA by forskolin (Fig. 3B, 1st and 3rd lanes). These results suggested that both endogenous 14-3-3 and exogenous 14-3-3-η associated with the α4β2 AChR complexes. In addition, in three independent experiments we also observed that both in the presence or absence of exogenous 14-3-3-η, the amount of α4 subunits immunoprecipitated by the anti-β subunit alone, we examined if 14-3-3-η, a chaperone protein, had a role in the early biogenesis of the α4 subunit. The α4 and the α4S441A subunits were separately cotransfected with or without 14-3-3-η cDNA into tsA201 cells. We studied the influence of the 14-3-3-η on the α4 or α4S441A 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 Fig. 4). Prior to activation of PKA, the presence of 14-3-3-η did not significantly alter the steady state levels of the α4 subunit. The α4 and the α4S441A subunits were separately cotransfected with or without 14-3-3-η cDNA into tsA201 cells. We studied the influence of the presence of 14-3-3-η on the α4 or α4S441A 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 Fig. 4). Prior to activation of PKA, the presence of 14-3-3-η did not significantly alter the steady state levels of the α4 subunit compared with the 1st lane or that of the α4S441A subunit (6th lane compared with the 5th lane). In the absence of 14-3-3-η, activation of PKA by forskolin did not significantly alter the steady state levels of the α4 subunit (3rd lane compared with the 1st lane) or the α4S441A subunit (7th lane compared with the 5th lane). 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 14-3-3-η (4th lane compared with the 2nd lane) in contrast to virtually no change in the steady state levels of the mutant α4S441A subunit under similar conditions (8th lane compared with the 6th lane). A similar result was obtained when cells were treated with fos-
In contrast, forskolin did not induce a statistically significant relative amount of primary anti-α4 subunit mAb bound to the surface AChRs when expressed alone or when coexpressed with 14-3-3ε and treated with forskolin (50 μM) 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 α4S441Aβ2 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 EC50 = 41 ± 3 μM (n = 7) and the wild-type α4β2 AChR coexpressed with 14-3-3ε with an EC50 = 64 ± 2 μM (n = 7). ACh activated the mutant α4S441Aβ2 AChR with an EC50 = 61 ± 3 μM (n = 7) and the mutant α4S441Aβ2 AChR coexpressed with 14-3-3ε with an EC50 = 55 ± 1 μM (n = 7). Following treatment with forskolin, the whole cell current activated by ACh and the EC50 values for AChR activation were not significantly different, these results suggested that 14-3-3ε 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 in transfected cells treated with and without forskolin (10 μM). Transfected cells were fixed with methanol/acetone and then sequentially immunostained for the α4 subunit followed by staining for 14-3-3ε as described under “Experimental Procedures.” 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 14-3-3ε 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 anti-α4α2 Ab to endogenous 14-3-3 proteins and no detectable nonspecific staining with the anti-α4α2 Ab (not shown). Thus, the colocalization results complemented the coimmunoisolation results and suggested that 14-3-3ε interacted with the α4 subunit and α4β2 AChRs primarily within

**Higher Surface Expression of Wild-type α4β2 AChRs Than Mutant α4S441Aβ2 AChRs**—To determine whether the interaction of 14-3-3ε with the α4 subunit alters the cell surface expression levels of α4β2 AChRs, we measured these levels for both wild-type α4β2 AChRs and mutant αS441Aβ2 AChRs using an enzyme-linked immunosassay. Because 14-3-3ε interacts with α4β2 AChRs following activation of PKA, we also examined if activation of PKA by forskolin (10 μM) altered cell surface expression levels of wild-type α4β2 AChRs and mutant αS441Aβ2 AChRs. The modified enzyme-linked immunosassay 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 immunoactivity 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 αS441Aβ2 AChRs (Fig. 5). Following treatment with forskolin (10 μM), 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 αS441Aβ2 AChRs. The 2-fold difference between the surface expression levels of the α4β2 AChRs and the αS441Aβ2 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 αS441A subunit CDNA due to differences in the quality of the DNA samples. Similar results in the absence of transfect ed exogenous 14-3-3ε (data not shown) are in keeping with our findings that the endogenous 14-3-3ε associated with α4β2 AChRs under these conditions.

**Functional Consequences of Coexpressing 14-3-3ε with Wild-type α4β2 AChRs and Mutant α4S441Aβ2 AChRs in Xenopus Oocytes**—We determined the functional consequences of the interaction of 14-3-3ε with α4β2 AChRs or αS441Aβ2 AChRs by studying their electrophysiological properties. AChR subunits were expressed from in vitro transcribed cRNAs microinjected into oocytes and currents elicited by 4-s applications of different concentrations of ACh recorded using two-electrode voltage clamp methodology. ACh elicited dose-dependent responses from both wild-type α4β2 AChRs and mutant αS441Aβ2 AChRs when expressed alone or when coexpressed with 14-3-3ε and treated with forskolin (50 μM) for 1 h. The bar graphs represent the normalized levels of AChR after subtraction 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.
AChRs coexpressed with 14-3-3

The significant 14-3-3 immunoreactivity detected with α4β2 AChRs immunopurified with two different mAbs to the α4β2 AChRs compared with 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) and 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, 14-3-3, 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 14-3-3 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 serine 441 is phosphorylated by PKA. By treating transfected α4β2 AChRs with forskolin, we demonstrated that 14-3-3-binding site motif is also present in the 413 and 450. By using site-directed mutagenesis we show that changing serine 441 to alanine nearly completely abolished the interaction of 14-3-3 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 PKA.

14-3-3 proteins have previously been shown to bind to the sequence motif (RSXXXP), 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 (Arg-Ser-Leu-Ser441-Val-Gln) between residues 413 and 450. By using site-directed mutagenesis we show that changing serine 441 to alanine nearly completely abolished the interaction of 14-3-3 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 14-3-3.

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 14-3-3 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 α4 subunit and not the mutant α4S441A subunit following activation of PKA by forskolin only in the presence of 14-3-3. Corresponding differences in the steady state levels of the α4β2 AChR and the α4S441Aβ2 AChRs were also observed and strongly suggested that 14-3-3 plays a role in early posttranslational events that govern subunit and α4β2 AChR stability.

The phosphorylation of the α4 subunit at serine 441 by PKA and its subsequent interaction with 14-3-3 alters cell surface α4β2 AChRs by increasing the α4 subunit and α4β2 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 α4β2 AChRs and its ability to bind 14-3-3 and lower surface expression levels of the mutant α4S441Aβ2 AChRs and their inability to bind 14-3-3. 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 α4S441Aβ2 AChRs. Similar results in surface expression levels following treatment with forskolin were observed in the absence of exogenous 14-3-3 and were most probably due to the observed ability of endogenous 14-3-3 proteins to interact with α4β2 AChRs.

Previously, it has been reported that activation of PKA by forskolin results in an −200% increase in cell surface expression of recombinant human α4β2 AChRs expressed in tsA201 cells (35). However, we do not observe such a large increase in surface expression of rat α4β2 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

We observed that activation of PKA significantly enhanced the interaction of 14-3-3 with unassembled α4 subunits and with assembled α4β2 AChR complexes. We have, however, failed to detect an increase in association of 14-3-3 with α4β2 AChRs in tsA201 cells following acute or chronic (24 h) exposure of AChRs to nicotine (data not shown). These results suggest that other intracellular processes, other than channel activity, possibly govern the interaction of 14-3-3 with the α4 subunit and α4β2 AChRs.

We have provided compelling evidence for a role of 14-3-3 in increasing the stability of the α4 subunit and α4β2 AChR under conditions that also correlate well with those that favor interaction of 14-3-3 with the α4 subunit. When α4 subunits are expressed alone, the wild-type α4 and mutant α4S441A 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 α4 subunit and not the mutant α4S441A subunit following activation of PKA by forskolin only in the presence of 14-3-3. Corresponding differences in the steady state levels of the α4β2 AChR and the α4S441Aβ2 AChRs were also observed and strongly suggested that 14-3-3 plays a role in early posttranslational events that govern subunit and α4β2 AChR stability.

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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 (36–45). 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 are more highly phosphorylated in the unassembled than in the assembled state indicating that phosphorylation precedes assembly and that phosphorylation/dephosphorylation mechanisms control the AChR subunit (36). Furthermore, using Torpedo AChR subunits expressed in mouse fibroblasts, it has been demonstrated previously 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 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 its 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 demonstrated previously to be localized appropriately 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 in its promoter (56) and as such its expression levels are likely to be regulated by changes in cellular levels of cAMP through the activation of the transcription factor cAMP-responsive element-binding protein. Thus we speculate that 14-3-3 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 α4 subunits might be better understood by modulation of the expression levels of 14-3-3γ in vivo.

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The Chaperone Protein 14-3-3\(\eta\) Interacts with the Nicotinic Acetylcholine Receptor \(\alpha 4\) Subunit: EVIDENCE FOR A DYNAMIC ROLE IN SUBUNIT STABILIZATION

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