Calnexin-dependent Enhancement of Nicotinic Acetylcholine Receptor Assembly and Surface Expression*

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The muscle-type nicotinic acetylcholine receptor (AChR)² is a pentameric membrane ion channel assembled in the endoplasmic reticulum from four homologous subunits by mechanisms that are insufficiently understood. Nascent AChR subunits were recently found to form complexes with the endoplasmic reticulum-resident molecular chaperone calnexin. To determine the contribution of this interaction to AChR assembly and surface expression, we have now used transient transfection of mouse AChR subunits and calnexin into non-muscle cells. Co-transfection of calnexin along with AChR subunits into COS and HEK 293 cells was found to enhance AChR subunit folding and assembly, and to decrease degradation rates of newly synthesized AChR α-subunits, resulting in elevated surface expression of assembled AChR. Moreover, inhibition of the interaction between endogenous calnexin and AChR by castanospermine resulted in decreased AChR subunit folding, assembly, and surface expression in muscle and HEK 293 cells. Together, these findings provide evidence that calnexin directly contributes to AChR biogenesis by promoting subunit folding and assembly.

Many of the transmembrane signaling molecules present in cell surface membranes are oligomeric proteins, and assembly of these proteins from constituent subunits is required for their functional expression. The nicotinic acetylcholine receptor (AChR), a ligand-gated ion channel which mediates nerve to muscle transmission, is a pentamer which is assembled in the endoplasmic reticulum (ER) from four distinct subunits coded by different genes in the stoichiometry α₂βγδ (1–4). The nascent AChR subunits are cotranslationally inserted into the ER membrane in a characteristic orientation with four transmembrane stretches, and with both the long amino-terminal domain and the short carboxyl terminus situated in the lumen of the ER. In the ER the newly translated subunits undergo post-translational modifications, including N-linked glycosylation and disulfide bond formation, as well as a sequence of folding events culminating in the acquisition of an assembly-competent conformation (5). In the case of α-subunits, this conformational maturation results in the appearance of a specific epitope termed the main immunogenic region, which is recognized by conformation-specific antibodies to AChR, as well as high affinity binding sites for the neurotoxin α-bungarotoxin (Bgt) (1, 6). These sites appear on α-subunits prior to their assembly with other subunits in contrast, the binding sites for AChR agonists such as acetylcholine and carbamylcholine arise only after the assembly of α- with γ- or δ-subunits (7). Since in the assembled AChR these agonist sites partially overlap with the neurotoxin-binding sites (4), the fraction of Bgt binding that is preventable by the presence of carbamylcholine can serve as a measure of AChR assembly (8). Only the assembled pentameric AChRs exit the ER and reach the cell surface, whereas unassembled or misfolded subunits and partial assembly intermediates are retained and degraded intracellularly (9–11), as is the case with other oligomeric proteins assembled in the ER (12, 13).

The expression of recombinant AChR subunits in heterologous cell lines has been used to address a number of fundamental questions relating to AChR assembly, such as the order in which the subunits are assembled (10, 14), the role of post-translational modifications in the regulation of AChR assembly (15, 16), and the identity of domains that form contacts between the subunits (8). However, this approach is hampered by the low levels of correctly assembled AChR at the surface of transfected cells. This may reflect the tendency of nascent subunits to misfold and aggregate, a problem frequently encountered in the expression of recombinant proteins in various transfection systems. Under normal conditions, newly synthesized membrane proteins are thought to be protected from misfolding by interactions with ER-resident molecular chaperones (17, 18). Calnexin, an ER constituent that is itself a transmembrane protein (19–21), has recently been shown to mediate folding and assembly of two oligomeric membrane glycoproteins: major histocompatibility complex class II molecules (22) and influenza hemagglutinin (23). We have recently observed that calnexin forms transient complexes with newly synthesized AChR α-subunits in muscle cells, as well as in COS cells transiently transfected with this subunit (24). Other transfected AChR subunits were also found to interact with calnexin in HEK 293 cells (25, 26). In muscle cells, the timing of this interaction appears to be precisely regulated, with binding occurring immediately upon subunit translation and dissociation taking place prior to AChR assembly (24, 27). This time course is consistent with the possibility that calnexin assists AChR assembly by facilitating the folding of nascent subunits into an assembly-competent conformation. However, direct functional evidence that calnexin promotes maturation and...
Calnexin Facilitates AChR Subunit Folding and Assembly

assembly of AChR subunits in the ER has been lacking.

In the present study we have examined the contribution of calnexin to AChR biogenesis in two ways: first, we took advantage of the inefficient folding and assembly of recombinant AChR subunits in transfected cells to determine if co-transfection of exogenous calnexin into these cells can increase AChR expression. Second, the ability of the glucose compromising inhibitor castanospermine (CAS) to block the interaction of endogenous calnexin with its substrates (28) was utilized to examine the effect of this block on AChR biogenesis.

EXPERIMENTAL PROCEDURES

Cells and Reagents—COS cells (African green monkey kidney cells) and HEK 293 cells (human embryonic kidney cells) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. C2C12 mouse muscle cells were grown in DMEM supplemented with 10% fetal bovine serum, and induced to differentiate by replacing this medium with DMEM, 2% horse serum. Muscle primary cultures were prepared from breast muscle of 12-day-old chick embryos as described previously (29), plated on collagen-coated dishes at initial densities of 6 × 10^5 cells/100-mm culture dish, and grown in DMEM supplemented with 10% horse serum and 2% chick embryonic extract. Calnexin labeling was performed using [35S]methionine (specific activity 1175 Ci/mmol) and [35S]-bungarotoxin (specific activity 13–15 μCi/μg). Calnexin was purified from 20% liver cytosol by affinity chromatography on CNBr-activated blue gel (30). The amounts of calnexin in the supernatants were determined using the highly specific AChR ligand [125I]-Bgt. The amounts of cell surface and intracellular AChR were determined as described by Kreienkamp et al. (8). For quantification of cell surface AChR, cultures were washed once with DMEM, then cells were solubilized in phosphate-buffered saline in the presence of [125I]-Bgt (30). The amounts of cell surface AChR were quantified in these metabolically labeled cells using the highly specific AChR ligand [125I]-Bgt. To measure total folded α-subunit, labeling was carried out as above, except that cells were incubated with [125I]-Bgt in saponin permeabilization buffer (10 mM sodium phosphate, pH 7.5, 10 mM EDTA, 0.1% bovine serum albumin, 0.5% saponin). Total assembled AChR was estimated as the component that is prevented from binding to the C2C12 Muscle Cells, COS Cells, and HEK 293 Cells—

Transfections—Full-length cDNAs coding for mouse AChR subunits (cDNA for α-subunit) was obtained from Dr. J. J. M. Bergeron (McGill University, Montreal, Canada). Anti-HA antibody was obtained from Dr. D. Bar-Sagi (SUNY at Stony Brook, Stony Brook, NY). Transfections—Full-length cDNAs coding for mouse AChR subunits (cDNA for α-subunit) was obtained from Dr. Jim Boulter, Salk Institute, La Jolla, CA; cDNAs for β-, γ-, and δ-subunits were provided by Drs. James Patrick, Baylor College of Medicine, Houston, TX; and Thomas (National Research Council of Canada, Montreal, Canada) and was subcloned into pBK/cytomegalovirus expression vector (Stratagene, La Jolla, CA). The HA Erk2 mitogen-activated protein kinase (MAP kinase) expression vector under the control of cytomegalovirus promoter was purchased from Stratagene (La Jolla, CA). The HA Erk2 mitogen-activated protein kinase (MAP kinase) expression vector under the control of cytomegalovirus promoter was obtained from Dr. J. J. M. Bergeron (McGill University, Montreal, Canada) and was subcloned into pBLCMV-galactosidase expression vector (StaDeW, Stony Brook, NY). Transfections of COS and HEK 293 cells for transient expression of AChR was carried out by DNA-calcium phosphate precipitation as described (31). Briefly, 60-mm dishes of cells at 50–60% confluence were incubated for 16–20 h at 37 °C with 5–10 μg of the cDNA to be transfected in a mixture containing CaCl$_2$ and HEPES-buffered saline solution. In cases when transfections were carried out with all four AChR subunits, the ratio of cDNAs encoding each subunit was 2:1:1:1. To rule out the possibility that any differences in transfection efficiencies contribute to the observed effects of co-transfected calnexin, the Escherichia coli β-galactosidase was added to the transfection mixture and its expression was measured by colorimetric assay as described (31).

Metabolic Labeling and Immunoprecipitations—Cultures were incubated in methionine-free DMEM for 1 h, then labeled at 37 °C for 2 h with a mixture of [35S]methionine and [35S]cysteine (for specific activities see figure legends) in methionine-free DMEM. ChR subunits were isolated by washing cells once with DMEM, followed by incubation in DMEM supplemented with 5 mM 1-methionine. Incubations were terminated by two washes with ice-cold Dulbecco’s phosphate-buffered saline, and subsequently cultures were harvested by scraping and extracted for 30 min at 4 °C in STE buffer (150 mM NaCl, 10 mM Tris-Cl, pH 7.4, 2 mM EGTA, 2 mM EDTA) containing 1 mg/ml aprotinin, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and supplemented with 1% Triton X-100. Extracts were clarified by centrifugation for 15 min at 12,000 × g in a microcentrifuge at 4 °C. The clarified supernatants were incubated at 4 °C with the specified antiserum for 3 h, then with protein-A-Sepharose beads (for anti-Bgt) or rabbit anti-rat antibody-coated protein-A-Sepharose beads (for mAb 61, mAb 35) for a further 1 h. The precipitates were washed five times with STE-Triton X-100 and fractionated on 10% SDS-polyacrylamide gels. Radioactive bands were visualized by radiofluorography and quantified by densitometry or PhosphorImaging.

Western Blotting—COS cells were transfected with all four AChR subunits or with HA-epitope-tagged MAP kinase in the absence or presence of calnexin cDNA. After 2 days cells were scraped into STE, 1% Triton X-100 buffer at 4 °C, and extracts were clarified as above. Aliquots of lysates from calnexin co-transfected and control cultures, normalized for the transfection efficiency, were fractionated by SDS-polyacrylamide gel electrophoresis. Proteins were then transferred onto nitrocellulose membranes, which were incubated either with anti-calnexin or anti-HA antibody. The blots were processed using the enhanced chemiluminescence technique (Amersham).

AChR Labeling—The amounts of cell surface and intracellular AChR were determined as described by Kreienkamp et al. (8). For quantification of cell surface AChR, cultures were washed once with DMEM, then cells were solubilized in phosphate-buffered saline in the presence of [125I]-Bgt, and labeling was carried out in the presence of carbamylcholine. To measure total folded α-subunit, labeling was carried out as above, except that cells were incubated with [125I]-Bgt in saponin permeabilization buffer (10 mM sodium phosphate, pH 7.5, 10 mM EDTA, 0.1% bovine serum albumin, 0.5% saponin). Total assembled AChR was estimated as the component that is prevented from binding to the C2C12 Muscle Cells, COS Cells, and HEK 293 Cells—

Comparison of AChR Assembly and Surface Expression in C2C12 Muscle Cells, COS Cells, and HEK 293 Cells—Fig. 1A compares AChR subunit synthesis, assembly, and surface expression in three cell types: C2C12 mouse muscle cells which normally express AChR upon differentiation, as well as COS and HEK 293 cells transiently transfected with the four (αβδ) mouse AChR subunits. To measure AChR α-subunit synthesis, equivalent amounts of each of these cell types were labeled for 1 h with [35S]cysteine/methionine mixture, then extracted and immunoprecipitated with mAb 61, an antibody directed against mouse AChR α-subunit. This approach showed substantial levels of AChR α-subunit synthesized in muscle cells (lane 2), and even higher levels in transfected COS cells and HEK 293 cells (lanes 3 and 4). In parallel experiments, assembled and surface AChR were quantified in these metabolically labeled cells using the highly specific AChR ligand Bgt. To measure total assembled AChR, cells were permeabilized with saponin prior to incubation with Bgt (8). Under these conditions Bgt can bind to both assembled AChR and unassembled but correctly folded α-subunits. To ascertain that the radiolabeled bound anti-Bgt immunoprecipitates shown in the middle panel of Fig. 1 corresponds predominantly to assembled α-subunits, the amounts of [125I]-Bgt binding in the presence and absence of 10 mM carbamylcholine were compared. Since the carbamylcholine-binding sites are comprised of domains from sets of 2 subunits (α-γ and α-δ), carbamylcholine will bind (and consequently block Bgt binding) to assembled α-subunit but not to unassembled α-subunit (8). The contribution of these unassembled subunits to
The total binding was not significant (<5%, data not shown). It should be noted that in contrast to the α-subunit which is clearly identifiable as a 40-kDa band, the other 3 subunits are difficult to resolve in [35S]methionine/labeled immunoprecipitates of assembled AChR, presumably due to their migration as diffuse bands, susceptibility to proteolysis, and the relatively high backgrounds in this region of the gel (15, 24, 29).

In replicate cultures, cell surface AChR was measured by the binding of Bgt to intact cells. After incubation with Bgt, cells were extracted and AChR-Bgt complexes were immunoprecipitated with anti-Bgt antibody. As can be seen in Fig. 1A, in C2C12 muscle cells during a 3-h chase period a significant proportion of the labeled α-subunit was assembled into AChR pentamers (lane 5), and reached the cell surface (lane 8). In contrast, in each of the transfected cell lines during the same interval only a small fraction of the total α-subunit synthesized underwent assembly with other subunits (lanes 6 and 7) and appeared at the plasma membrane (lanes 9 and 10). These results are quantified in Fig. 1B, which shows that the transfected HEK 293 and COS cells each display a substantially lower efficiency of AChR assembly and surface expression relative to the muscle cell line.

**Effect of Calnexin Co-transfection on AChR Assembly and Surface Expression**—To determine if the low levels of AChR assembly in transfected cells overexpressing the individual subunits reflect the exhaustion of endogenous calnexin, we measured the effect of co-transfecting calnexin cDNA on AChR assembly and surface expression. In the experiments shown in Fig. 2A, COS and HEK 293 cells were transfected with all four AChR subunits with or without calnexin, and 2 days later assembly was measured by the binding of 125I-Bgt to saponin-permeabilized cells, while surface AChR was quantified by 125I-Bgt binding to intact cells. As can be seen, the levels of both assembled and surface AChR were markedly higher in cultures co-transfected with calnexin, consistent with the possibility that AChR assembly in the transfected cells was limited by the availability of this molecular chaperone. In contrast, co-transfection with calnexin did not cause the translocation of unassembled α-subunit to the plasma membrane, since no appreciable surface Bgt binding was detected when calnexin was co-transfected into either COS or HEK 293 cells expressing α-subunit alone (Fig. 2A, first two bars in each panel). The overall levels of calnexin, as measured by Western blotting, were higher in calnexin-transfected cultures (Fig. 2C, top panel) (typically by 20–30%). Since the proportion of transfected cells in these experiments was between 5 and 10%, as determined by the β-galactosidase staining of fixed cultures (not shown), the levels of transferred calnexin in the transfected cells were estimated to significantly exceed (by 4–10-fold) those of endogenous calnexin. Together these results indicate that the increase in Bgt binding reflects specific stimulation of AChR biogenesis by co-transfected calnexin.

The effects of calnexin co-transfection on AChR assembly and surface expression were also examined by a second method: immunoprecipitation of Bgt-AChR complexes from [35S]methionine/cysteine-labeled COS cells that had been transfected with all four AChR subunits from [35S]methionine/cysteine-labeled COS cells that had been transfected with all four AChR subunits with or without calnexin. As can be seen in Fig. 2B, the amounts of both total (intracellular + surface) assembled AChR and surface AChR were increased in the cultures co-transfected with calnexin (lanes 4 and 6 versus lanes 3 and 5). Interestingly, the overall levels of radiolabeled AChR α-subunit accumulated during the 1-h labeling period were also higher in cells co-transfected with calnexin as compared with cultures transfected with AChR subunits but not with calnexin (Fig. 2B, compare lanes 1 and 2). The increased accumulation of α-subunit in calnexin co-transfected cells was also evident from the Western blot (Fig. 2C, middle panel). In contrast, the levels of a transfected cytoplasmic protein, MAP.
kinase, were not altered by co-transfection with calnexin (Fig. 2C, bottom panel), consistent with the known localization of calnexin to the ER and the restriction of its chaperone functions to secretory and membrane proteins. It is noteworthy that the enhancement by co-transfected calnexin of AChR assembly and surface expression appeared to be greater than the increase in AChR subunit levels in these cultures (Fig. 2B). Thus, calnexin may contribute to AChR expression in two ways: by stabilizing newly synthesized AChR subunits in the ER, and by mediating the assembly of these subunits into pentameric AChR expressed on the cell surface.

Effect of Calnexin Co-transfection on the Folding of AChR α-Subunit—Calnexin has been proposed to mediate the folding of nascent AChR subunits prior to their assembly into multi-subunit complexes based on the timing of its interaction with α-subunit in cultured muscle cells (24). To directly investigate the contribution of calnexin to the conformational maturation of AChR α-subunit, we have measured the ability of conformationally mature α-subunits to bind Bgt. As can be seen in Fig. 3, calnexin co-transfection leads to an increase in intracellular 125I-Bgt binding to α-subunit expressed both in COS and HEK 293 cells. Thus elevation of calnexin levels appears to facilitate the folding of nascent AChR α-subunits in the transfected cells.

Effect of Calnexin Co-transfection on the Degradation Rate of AChR α-Subunit—In the transfection experiments summarized above we have observed that calnexin has stimulatory effects on the folding and assembly of AChR α-subunit, as well as on the accumulation of newly made α-subunits in the ER. As net accumulation represents the difference between biosynthesis and degradation, we next determined the effect of co-transfected calnexin on the degradation rate of α-subunit. For this purpose, COS cells were transfected with all four AChR subunits or with HA-epitope-tagged MAP kinase levels. COS cells were transfected with all four AChR subunits with or without co-transfected calnexin, and after 2 days these cultures were pulse-labeled for 15 min with [35S]methionine/cysteine and chased for the specified periods in the absence of radioactive amino acids. At the end of the chase period, cells were extracted and immunoprecipitated with mAb 61. The intensities of the bands corresponding to the radiolabeled α-subunit in the transfected cells were quantified by scanning densitometry of autoradiographs. To compare the degradation rates in the absence and presence of co-transfected calnexin, the values at each time point were expressed as the proportion of the radio-labeled α-subunit immunoprecipitated immediately after the pulse. In a representative experiment shown in Fig. 4, the disappearance of α-subunit in COS cells showed first-order kinetics with a half-life of approximately 1.5 h. This value is
Calnixin Facilitates AChR Subunit Folding and Assembly

**FIG. 3.** Effect of calnexin co-transfection on the folding of AChR α-subunit. COS cells and HEK 293 cells were transfected with AChR α-subunit in the absence (open bars) or presence (shaded bars) of co-transfected calnexin and 2 days later the folding of α-subunit was determined by measuring 125I-Bgt binding to transfected cells permeabilized in saponin buffer, after subtraction of 125I-Bgt binding to non-transfected cultures. Values shown are the mean of three determinations ± S.D.

**FIG. 4.** Effect of calnexin co-transfection on the degradation rate of AChR α-subunit in transfected cells. COS cells transfected with all four AChR subunits were pulse-labeled with [35S]methionine/ cysteine mixture (200 μCi/ml) for 15 min, then chased for various intervals up to 90 min. Cells were extracted and immunoprecipitated with mAb 61. The amount of radiolabeled α-subunit at each time point was quantified by scanning densitometry and plotted versus chase time on a semi-logarithmic scale.

in good agreement with the catabolic half-life of mouse AChR α-subunit previously measured in COS cells (11) and in quail fibroblasts (15, 32). In the presence of co-transfected calnexin α-subunit degradation rate was diminished significantly (half-time of approximately 4 h), indicating that interaction with calnexin can stabilize nascent AChR subunits in the ER. This decrease in AChR α-subunit degradation is likely to account for the enhanced accumulation of α-subunit observed in calnixin co-transfected cells (see Fig. 2B, lanes 1 and 2; Fig. 2C, middle panel). Moreover, co-transfected calnexin had a similar stabilizing effect on cultures transfected with α-subunit alone (not shown).

Effect of Castanospermine Treatment on AChR Expression—In the experiments described thus far, co-transfection with calnexin was seen to enhance AChR biogenesis in cells that are inefficient in the assembly and surface expression of these receptors. We next examined the contribution of calnexin to AChR expression in primary cultures of embryonic chick myotubes, a cell system that assembles AChR with high efficiency (29). In these experiments we utilized the glucosidase inhibitor CAS, that was recently shown to interfere with the binding of calnexin to nascent glycoproteins (28). Chick muscle cells were labeled with [35S]methionine/cysteine for 1 h in the absence or presence of 1 mM CAS, and the association of AChR α-subunit with calnexin was measured by sequential immunoprecipitation as described (24). As can be seen in Fig. 5A, α-subunits synthesized in the presence of CAS migrated at a slightly higher molecular weight than α-subunits from untreated cultures, reflecting the block in glucose trimming by the inhibitor (Fig. 5A, compare lanes 2 and 4). We observed that the association of calnexin with these untrimmed α-subunits was strongly reduced (by approximately 80% as determined by scanning densitometry) in comparison with normally processed α-subunits (lanes 3 and 5).

To determine the effects of CAS treatment on AChR biogenesis, muscle cultures were pulse-labeled with [35S]methionine/cysteine for 1 h in the presence or absence of CAS, and either harvested directly after the pulse to measure the amount of α-subunits synthesized or chased for 3 h to allow sufficient time for AChR assembly and surface expression. Myotube extracts were immunoprecipitated with anti-chick AChR α-subunit antibody to determine total α-subunit levels (Fig. 5B, lanes 1 and 2), mAb 35 to monitor α-subunit folding (lanes 3 and 4), anti-

chick AChR δ-subunit antibody to measure assembly (lanes 5 and 6), or Bgt-anti-Bgt to measure AChR appearance at the cell surface (lanes 7 and 8). As can be seen in Fig. 5B, the folding, assembly, and surface expression of AChR were all significantly inhibited in muscle cells treated with CAS, whereas the total amounts of α-subunit accumulation were not affected.

To verify these findings we also measured the effects of CAS treatment on AChR assembly and surface expression using 125I-Bgt binding to permeabilized and intact muscle cells, respectively (Fig. 5C). In these experiments 2-day-old muscle cultures were exposed to CAS for 16 h during the period of maximal accumulation of AChR. Cells were then suspended and incubated with 125I-Bgt for 2 h. Under these conditions the number of surface and intracellular Bgt-binding sites in CAS-treated muscle cells was decreased by approximately 30% as compared with untreated cultures. To determine if the surface expression of transfected AChR in nonmuscle cells is comparably susceptible to CAS treatment, HEK 293 cells transfected with all four AChR subunits were exposed to CAS for 2 days starting at 16 h post-transfection. Surface AChR was then measured by 125I-Bgt binding to intact cells. As shown in Fig. 5D, CAS treatment diminished surface AChR levels by approximately 30%, i.e. to the same extent as in chick muscle cells. Furthermore, the CAS treatment eliminated the stimulation of AChR surface expression by cotransfected calnexin in the HEK 293 cells (data not shown).

**DISCUSSION**

In this study two lines of evidence point to the direct participation of calnexin in the control of muscle AChR biogenesis. First, co-transfection of calnexin cDNA along with cDNAs for all four subunits of muscle-type AChR into COS and HEK 293 cells was found to produce a significant increase in the folding,
assembly, and surface expression of AChR, as compared with replicate cultures transfected with the four AChR subunits only. A second group of experiments in which the glucose trimming inhibitor CAS was used to block calnexin-AChR interaction, demonstrated that the efficiency of AChR subunit folding, assembly, and surface appearance is diminished when calnexin binding is impaired.

The current observation that the folding and assembly of AChR subunits can be stimulated by co-transfected calnexin indicates that the availability of calnexin is limiting for the efficient AChR oligomerization in COS and HEK 293 cells transfected with the four AChR subunits. Thus, the low efficiency of surface expression of assembled AChR in transfected cells may be due to the fact that the high levels of expression of the transfected subunits exceeded the capacity of the ER molecular machinery to process and assemble these polypeptides. It is noteworthy in this context that despite the enhancement caused by calnexin, the extent of AChR assembly in the transfected cells remained lower than in muscle cells expressing endogenous AChR. This implies that in addition to calnexin, other ER-resident proteins that contribute to AChR biogenesis may be exhausted by the overexpression of transfected subunits. Aside from calnexin, other candidate ER-resident proteins that may participate in AChR biogenesis include peptidyl-prolyl isomerase which was recently reported to facilitate disulfide isomerase, an enzyme shown to increase the efficiency of folding of secretory proteins in yeast (34); and immunoglobulin heavy chain-binding protein, a molecular chaperone shown to interact with AChR subunits in transfected cells (35, 14). In addition, it is possible that there exist as yet uncharacterized muscle-specific mechanisms that contribute to efficient oligomerization of AChR.

In our experiments higher amounts of AChR α-subunits accumulated in cells co-transfected with calnexin as compared with cells transfected only with the AChR subunits. This effect may reflect, at least in part, the decreased rate of AChR α-subunit degradation measured in calnexin co-transfected cells (Fig. 4). A similar capacity of calnexin to protect from degradation has been reported for other nascent proteins, including subunits of T cell receptors (36), major histocompatibility complex class I (22) and class II (37) complexes, as well as influenza hemagglutinin (23). However, the difference in the degradation kinetics as calculated from the semilogarithmic plots shown in

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**Fig. 5. Effects of castanospermine treatment on AChR α-subunit interaction with calnexin, folding, assembly, and surface expression.** A, chick myotubes were incubated with (lanes 4 and 5) or without (lanes 1, 2, and 3) 1 mM CAS for 1 h in methionine-free medium, then [35S]methionine/cysteine mixture (400 μCi/ml) was added for an additional 1 h. Cells were then extracted and sequentially immunoprecipitated with anti-calnexin antibody followed by non-immune antiserum (lane 1), anti-chick AChR α-subunit antibody twice in sequence (lanes 2 and 4), or with anti-calnexin antibody followed by anti-chick AChR α-subunit antibody (lanes 3 and 5). B, chick myotubes were incubated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) 1 mM CAS for 1 h in methionine-free medium, then [35S]methionine/cysteine mixture (200 μCi/ml) was added for 1 h. Cells were either harvested directly for immunoprecipitations with anti-chick AChR α-subunit antibody (lanes 1 and 2) or chased for 3 h with (lanes 4, 6, and 8) or without 1 mM CAS (lanes 3, 5, and 7). For immunoprecipitation of surface AChR (lanes 7 and 8), cells were incubated with [125I]-Bgt (10 nM) during the last 1 h of chase period. Cells were then harvested in STE, 1% Triton X-100 buffer and immunoprecipitated with mAb 35 (lanes 1, 3, 5, and 7). A similar capacity of calnexin to protect from degradation has been reported for other nascent proteins, including subunits of T cell receptors (36), major histocompatibility complex class I (22) and class II (37) complexes, as well as influenza hemagglutinin (23). However, the difference in the degradation kinetics as calculated from the semilogarithmic plots shown in
Calnexin Facilitates AChR Subunit Folding and Assembly

Fig. 4 does not appear to be sufficiently large to account for the increase in levels of AChR α-subunit measured in calnexin-cotransfected cells after labeling intervals as brief as 60 (Fig. 2B, lanes 1 and 2) or 15 min (Fig. 4, first lanes in both panels). Thus, a possibility exists that calnexin diverts into the folding pathway a proportion of newly synthesized α-subunits, which otherwise undergo rapid degradation during or shortly after translation, as recently suggested for H-2 Dα major histocompatibility complex class I heavy chains (22).

Although the means by which calnexin exerts its protective function are yet to be defined, several possible mechanisms may be envisioned. First, the binding of calnexin may prevent translocation of newly synthesized AChR subunits to sites of degradation. A growing body of evidence indicates that in the case of several secretory pathway proteins these sites of degradation are the cytosolic 26 S proteasomes (38–40), and thus degradation involves translocation of these integral membrane or ER luminal proteins into the cytosol (41–43).

In preliminary experiments supporting this possibility we have observed that degradation of AChR α-subunits in transfected COS cells was slowed in the presence of the proteasome inhibitors lactacystin and N-acetyl-leu-leu-norleucinal (44, 45). Second, by promoting the folding of nascent subunits, calnexin may decrease the proportion of misfolded AChR subunits that are targeted for degradation and increase the pool of subunits in the productive folding/assembly pathway. Third, as has been proposed for T-cell receptors, the subunits of oligomeric proteins in the secretory pathway may contain regions coding for degradation that are only exposed on unassembled monomers (46). Calnexin may act as a surrogate subunit and shield these regions in newly synthesized AChR subunits, thereby preventing their ER-associated proteolysis during the interval preceding AChR oligomerization. The stabilization of nascent monomeric AChR subunits through protein-protein interactions in the ER has been documented in the case of interaction with other AChR subunits. Thus, the formation of AChR assembly intermediates has been shown to decrease the degradation rate of α-subunits (11, 26, 47). In the case of AChR α-subunit-calnexin interaction the degree of stabilization, although significant, appears to be less pronounced than that achieved through association with other subunits (11, 26). This may reflect the transient nature of interaction with the molecular chaperone as opposed to the more stable complexes formed between the subunits.

Using cultured muscle cells, we have recently shown that AChR assembly can be blocked as a result of impaired subunit folding in the presence of dithiothreitol, a reducing agent which prevents disulfide bond formation on nascent subunits (27). We have now found that AChR assembly is also impaired upon inhibition of another post-translational modification of nascent AChR subunits, the trimming of glucose residues on N-linked oligosaccharides, induced by CAS. In these experiments, CAS treatment strongly inhibited calnexin-AChR α-subunit interaction, as has been shown for other calnexin substrates (28), and this block is assumed to be responsible for the decreased efficiency of AChR subunit folding, assembly, and surface expression in CAS-treated muscle cells. Furthermore, the observation that CAS exerts highly similar effects on surface expression of transfected AChR in HEK 293 cells lends support for the role of calnexin in AChR expression. However, the possibility that CAS has additional effects on AChR expression, for example, by impairing the activity of a soluble ER-resident molecular chaperone calreticulin that shares structural homology and lectin-like binding properties with calnexin (29), has not been ruled out by these experiments. It is significant that under these conditions a proportion of untrimmed AChR α-subunits become assembled and appear at the cell surface, indicating that AChR biogenesis can proceed without the participation of calnexin, but at markedly lower efficiency. The persistence of AChR assembly in CAS-treated cells is in agreement with a recent study utilizing an in vitro translation system in which α-subunit folding, as measured by acquisition of Bgt binding, and its assembly with δ-subunits, were reported to occur in the presence of CAS (48).

In contrast to our present findings, no decrease in the extent of α-subunit folding and assembly by CAS treatment was detected in this in vitro system. This apparent discrepancy is presumably due to the differences between the two experimental systems: it is possible that the high levels of AChR obtainable with intact cells are required to measure the contribution of glucose trimming to AChR expression. In addition, the use of intact cells is advantageous in that it allows measurement of the effect of CAS treatment on AChR surface expression.

In summary, the present study shows that calnexin facilitates AChR assembly and surface expression by promoting the correct folding of subunits for efficient oligomerization. Fuller characterization of the molecular components and functional topology of the ER would further the understanding of the cellular control of expression of AChR and other oligomeric membrane proteins.

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Calnexin Facilitates AChR Subunit Folding and Assembly

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