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Syntrophins Regulate α_{1D}-Adrenergic Receptors through a PDZ Domain-mediated Interaction*

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To find novel cytoplasmic binding partners of the α_{1D}-adrenergic receptor (AR), a yeast two-hybrid screen using the α_{1D}-AR C terminus as bait was performed on a human brain cDNA library. α-Syntrophin, a protein containing one PDZ domain and two pleckstrin homology domains, was isolated in this screen as an α_{1D}-AR-interacting protein. α-Syntrophin specifically recognized the C terminus of α_{1D}- but not α_{1A}- or α_{1B}-ARs. In blot overlay assays, the PDZ domains of syntrophin isoforms α, β1, and β2 but not γ1 or γ2 showed strong selective interactions with the α_{1D}-AR C-tail fusion protein. In transfected human embryonic kidney 293 cells, full-length α_{1D}- but not α_{1A}- or α_{1B}-ARs co-immunoprecipitated with syntrophins, and the importance of the receptor C terminus for the α_{1D}-AR/syntrophin interaction was confirmed using chimeric receptors. Mutation of the PDZ-interacting motif at the α_{1D}-AR C terminus markedly decreased inositol phosphate formation stimulated by norepinephrine but not carbachol in transfected HEK293 cells. This mutation also dramatically decreased α_{1D}-AR binding and protein expression. In addition, stable overexpression of α-syntrophin significantly increased α_{1D}-AR protein expression and binding but did not affect those with a mutated PDZ-interacting motif, suggesting that syntrophin plays an important role in maintaining receptor stability by directly interacting with the receptor PDZ-interacting motif. This direct interaction may provide new information about the regulation of α_{1D}-AR signaling and the role of syntrophins in modulating G protein-coupled receptor function.

α_1-Adrenergic receptors (α_1-ARs)[2] are G protein-coupled receptors that mediate various important physiological functions of norepinephrine (NE) and epinephrine, particularly in the cardiovascular system where they are responsible for regulating vascular tone and peripheral resistance. Three α_1-AR subtypes have been cloned (α_{1A}-, α_{1B}-, and α_{1D}-AR) and display differences in sequence homology and affinities for subtype selective ligands (1). Upon agonist stimulation, all three α_1-AR subtypes signal through $G\alpha_{q/11}$ to increase phospholipase C activity and intracellular Ca^{2+} mobilization (1, 2). In addition, recent studies using α_1-AR transgenic and knock-out mice have now revealed that all three α_1-AR subtypes are important for the regulation of blood pressure (2, 3). Therefore, it remains unclear if specific functional differences exist between the α_1-AR subtypes.

Increasing evidence now suggests that α_1-AR subtypes display differences in their ability to interact with specific protein binding partners. The first α_1-AR subtype-selective binding partner identified was tissue transglutaminase II, which selectively associates with α_{1B}- and α_{1D}-ARs (4, 5). Since then, other proteins found to selectively associate with α_1-ARs include gC1qR (6), adaptor protein complex-2 (7), regulators of G protein signaling-2 (8), and spinophilin (9). These interactions were shown to be important for the signaling (8, 9), trafficking (6), and internalization (7) properties of the α_1-ARs. Therefore, these differential interactions may contribute to subtype-specific differences between the members of the α_1-AR family.

Previously, the α_{1D}-AR was the least studied of the α_1-AR subtypes due to difficulties in obtaining significant cell surface expression and poor signaling in heterologous systems. Recent studies have reported that this is due to the primary intracellular localization of this receptor (10, 11). Subsequent findings indicated that N-terminal truncation (11, 12) or heterodimerization with α_{1B}-ARs (13, 14) or β_2-ARs (15) promotes α_{1D}-AR cell surface expression and increases coupling to functional responses. However, it remains unknown if there are any α_{1D}-AR accessory proteins involved in α_{1D}-AR function and/or expression.

In this study, we have identified several closely related syntrophin family isoforms (α, β1, and β2) as novel α_{1D}-AR binding partners using a combination of yeast two-hybrid screening and biochemical techniques. We report that syntrophins directly interact with α_{1D}-ARs through a PDZ domain-mediated interaction. The specificity of this association and its potential role in regulating α_{1D}-AR expression and signaling were examined.

EXPERIMENTAL PROCEDURES

Materials and Reagents—Materials used in this study were obtained from the following sources. Components for yeast two-hybrid screening (Clontech, Palo Alto, CA): YPD agar and YPD broth (Qbiogene, Irvine, CA); 425–600 μM glass beads (acid-washed, G8772), anti-FLAG M2 affinity gel (A2220), and horseradish peroxidase (HRP)-conjugated anti-FLAG M2 antibody (Sigma-Aldrich). Cell culture media: trypsin (Mediatech, Herndon, VA), fetal bovine serum, Lipofectamine 2000, 4–20% Tris-glycine SDS-PAGE gel (Invitrogen); goat anti-mouse κ HRP-conjugate (Southern Biotechnology Associate, Birmingham, AL); human embryonic kidney 293 (HEK293) cells (ATCC, Manassas, VA); *n*-dodecyl-β-D-maltoside (Calbiochem); QIAprep Spin Miniprep kit (Qiagen, Valencia, CA); ProTran nitrocellulose (Schleicher & Schuell); ECL (PerkinElmer Life Sciences); HRP-conjugated S protein (Novagen, San Diego, CA); mouse anti-syntrophins antibody (MA1–745, Affinity BioReagents, Golden, CO).

Yeast Two-hybrid Screening—Plasmid pGBKT7/α_{1D}-C-tail (aa 480–572) was used as bait to screen a human brain pretransformed cDNA library (in pACT2) by using the standard yeast mating protocol stated in

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[2] The abbreviations used are: AR, adrenergic receptor; NE, norepinephrine; HRP, horseradish peroxidase; HA, hemagglutinin; HEK, human embryonic kidney; PDZ, PSD95/Discs-large/ZO-1 homology; RT, room temperature; PSD95, post-synaptic density protein of 95 kDa; MAGI2, membrane-associated guanylate kinase inverted 2; PH, pleckstrin homology; aa, amino acids; GST, glutathione *S*-transferase; InsP, inositol phosphate; hα-syn, human α-syntrophin.

the manual. Yeast were plated on high stringency selective medium (SD/−Leu/−Trp/−His/−Ade) and incubated for 7 days at 30 °C. Positive colonies were restreaked on selective medium (SD/−Leu/−Trp/−His/-Ade). Yeast DNA extracts were obtained by using QIAprep Spin Miniprep kit supplemented with 250 μl of 425–600 μM glass beads in buffer P1 by vortexing for 5 min. Library plasmid DNA was rescued from positive colonies by transforming yeast DNA extracts into *Escherichia coli* TOP 10 F′ cells and selected by 30 μg/ml ampicillin. Meanwhile, yeast DNA extracts were used as the template for PCR using MATCHMAKER 5′AD and 3′AD LD-insert screening amplimers as primers and subjected to further sequence analysis. To test the specificity of the interaction, isolated library cDNAs were co-transformed into yeast strain AH109 together with a bait plasmid, either pGBKT7/α_{1D}-C-tail, empty vector pGBKT7, or other plasmids as indicated. Transformed yeast were subjected to growth tests on high stringency selective medium.

Plasmid Construction—Mouse α-syntrophin in pBluescript II SK(−) was kindly given by Dr. Stanley Froehner (University of Washington, Seattle, WA). α-Syntrophin in pDT mammalian expression plasmid was constructed by cloning an α-syntrophin fragment from pBluescript/α-syntrophin into HindIII/BamHI sites of pDT vector. The N-terminal FLAG-tagged or HA-tagged α_1-AR constructs were constructed as previously described (12, 16). The α_{1D}-AR construct with the PDZ-interacting motif substituted with alanine residues was generated by PCR using the full-length α_{1D}-AR as template and the primers CAACCGCCACCTGCAGACCGTCACCAACTA (forward) and GCCGACTACAGCAACCTAGCAGCAGCAGCTGCTTAAACGCGTGCT (reverse), digested with AgeI and MluI, and ligated with HA-Ntrα_{1D} construct (Δ1–79 truncation) (12) to replace the corresponding normal C-terminal domain. The resultant α_{1D}-AR construct was sequenced to confirm the alanine substitution at the PDZ-interacting motif. The chimeric α_{1B}-AR construct with the α_{1D}-AR C terminus was constructed by the following. A silent mutation forming an EcoRI site (GAATTC) was introduced to the conserved EFK motifs at the α_{1B}-AR (1062G→A) and the α_{1D}-AR construct (1224G→A), respectively. The C-terminal regions of those two mutated constructs were swapped by EcoRI and MluI to generate the C-terminal chimeric receptor constructs.

Fusion Protein Construction—To construct GST-tagged receptor C-terminal fusion protein plasmids, β_1-C-tail (the C-terminal 100 aa of the human β_1-AR (17)) and α_{1D}-C-tail (the C-terminal 93 aa of the human α_{1D}-AR) were amplified by PCR and then subcloned into pGEX-4T1 using the EcoRI and XhoI sites. To construct hexahistidine-tagged and S protein-tagged PDZ domain fusion protein plasmids, α-Syn-PDZ (mouse α-syntrophin, 79–228 aa) was amplified by PCR and then inserted into pET30a at the BamHI and XhoI sites, whereas β1-Syn-PDZ (mouse β1-syntrophin, 106–252 aa), β2-Syn-PDZ (human β2-syntrophin, 111–265 aa), γ1-Syn-PDZ (mouse γ1-syntrophin, 52–201 aa), and γ2-Syn-PDZ (mouse γ2-syntrophin, 68–215 aa) constructs were prepared by PCR and inserted into pET30a at the EcoRI and XhoI sites. PSD95-PDZ3 (rat PSD 95, 307–446 aa) and membrane-associated guanylate kinase inverted-2 (MAGI2)-PDZ1 (human MAGI2, 446–571 aa) were generated as previously described (17).

Blot Overlay Assays—The interaction between the GST-tagged receptor C-terminal fusion proteins and His_6/S-tagged PDZ domain fusion proteins was assayed via blot overlay assays. 2 μg of purified His_6-tagged syntrophin PDZ domain fusion proteins were run on a 4–20% Tris-glycine SDS-PAGE gel for 1.5–2 h at 125 V and then transferred to ProTran nitrocellulose. The blot was blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST) consisting of 5% nonfat milk for 1 h at room temperature (RT), subsequently incubated with 25 nM GST-tagged α_{1D}-C-tail fusion proteins containing 2% nonfat milk for at least 1 h at RT, washed 3 times with TBST, incubated at RT with monoclonal anti-GST antibody, washed 3 times with TBST, and then incubated with a HRP-conjugated anti-mouse IgG secondary antibody. After 6 washes with TBST, bands were visualized with ECL. PSD95-PDZ3 and MAGI2-PDZ1 fusion proteins were used as controls. In other experiments 2 μg of purified GST or GST fusion proteins as indicated were run on an SDS-PAGE gel and overlaid with 25 nM His_6/S-tagged indicated PDZ domain fusion proteins. Interaction was detected by HRP-conjugated S protein and ECL.

Cell Culture and Transfection—HEK293 cells were propagated in Dulbecco's modified Eagle's medium (4.5 g/liter glucose) plus 10% heat-inactivated fetal bovine serum, 100 mg/liter streptomycin, and 10^5 units/liter penicillin at 37 °C in a humidified atmosphere with 5% CO_2. For transient transfection, 8 μg of indicated plasmid DNA was mixed with Lipofectamine2000 and serum-free medium at RT for 20 min and added to HEK293 cells growing in a 150-mm tissue culture plate. Cells were harvested 48–72 h after transfection for further experimentation. For stable transfection cells were selected in the presence of 400–800 μg/ml Geneticin.

Membrane Preparation—For radioligand binding, cells grown on 150-mm tissue culture plates were harvested in phosphate-buffered saline (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4), and membrane preparations were prepared as previously described (18). For immunoprecipitation, cells were collected by centrifugation at 30,000 × *g* for 20 min, and membrane preparations were prepared as previously described (13).

Immunoprecipitation and Immunoblotting—Membrane preparations were solubilized by 2% *n*-dodecyl-β-D-maltoside, and the supernatant was incubated with either anti-FLAG affinity gel or anti-HA affinity matrix in 0.2% *n*-dodecyl-β-D-maltoside prepared in 1× buffer (25 mM HEPES, 150 mM NaCl, pH 7.4, 5 mM EDTA) with a protease inhibitor mixture (1 mM benzamidine, 3 μM pepstatin, 3 μM phenylmethylsulfonyl fluoride, 3 μM aprotinin, and 3 μM leupeptin) overnight at 4 °C. An aliquot of 50 μl of supernatant was incubated with 4× Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.025% bromphenol blue, and 5% β-mercaptoethanol) to examine expression of proteins in the solubilized fraction. The next day the affinity gel/matrix was collected by centrifugation, washed with 1× buffer 3 times at 4 °C, and eluted with an equal volume of 4× Laemmli sample buffer. Immunoprecipitated samples were run on 4–20% Tris-glycine SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 5% nonfat dried milk in TBST buffer at RT, incubated with HRP-conjugated anti-FLAG M2 antibody (1:600) or anti-HA-mouse antibody (1:5000) at RT, washed with TBST and detected with ECL or incubated with anti-mouse secondary antibody (1:5000) and then detected with ECL. For overexpression of α-syntrophin with HF-α_{1D}-AR and ΔPDZ-HF-Ntrα_{1D}-AR, immunoprecipitation was done as previously described (19).

Radioligand Binding—Receptor density was determined by saturation binding assays as previously described (18).

^{3}H-Labeled Inositol Phosphate (InsP) Formation—Receptor function in transfected HEK293 cells was measured by [^{3}H]inositol phosphate (InsP) formation upon NE stimulation as previously described (18). 10^{-4} M carbachol was used as a control.

Data Analysis—Data were expressed as the mean ± S.E. of results obtained from the indicated number of observations. For saturation binding assays, K_D and B_{max} were calculated by nonlinear regression using Prism (GraphPad).

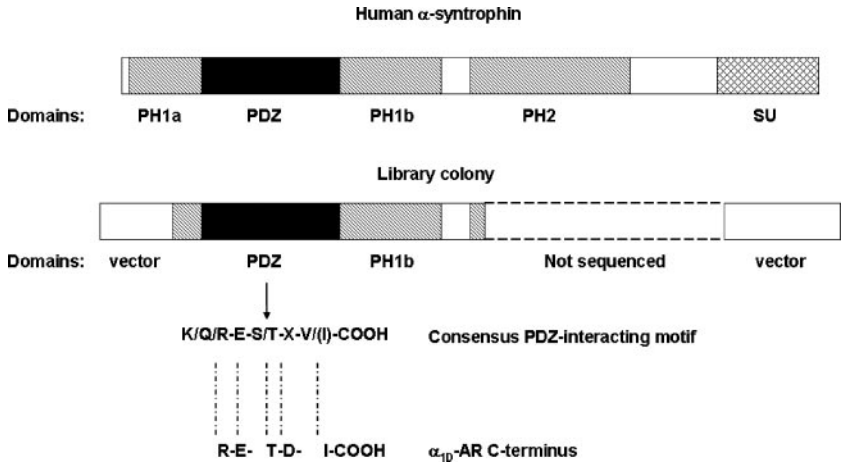

FIGURE 1. **Structure of the α-syntrophin clone identified from a yeast two-hybrid screen using the human α_{1D}-AR C terminus as bait.** A fragment of α-syntrophin with an intact PDZ domain was identified as a novel binding partner for the α_{1D}-AR. The comparison between the consensus sequence preferred for binding by syntrophins and the putative PDZ-interacting motif at the α_{1D}-AR C terminus is shown at the *bottom*.

RESULTS

α-Syntrophin Is a Specific Binding Partner for the α_{1D}-AR C Terminus—To identify novel α_{1D}-AR-associated proteins, a human α_{1D}-AR partial C terminus (α_{1D}-C-tail) was used as bait to screen a human brain pretransformed cDNA library. From a total of 3×10^5-independent diploids screened, 9 positive clones were obtained. One positive clone was identified as a gene fragment of human α-syntrophin (hα-syn). This fragment contained an intact PDZ domain and is displayed in schematic form in Fig. 1. The α_{1D}-AR is known to contain a putative PDZ-interacting motif at the distal end of its C-tail (^{568}RETDI^{572}) that is highly homologous to the conserved PDZ-interacting motif ((K/Q/R)E(S/T)*X*(V/I)) previously demonstrated to be recognized by syntrophins (20–22). The specificity of the interaction between hα-syn and α_{1D}-C-tail was confirmed by further yeast two-hybrid analysis. The hα-syn library construct was co-transformed into yeast strain AH109 with individual baits α_{1A}-, α_{1B}-, or α_{1D}-C-tails. Transformed yeast containing the indicated bait and hα-syn (Fig. 2*A*) were subjected to growth tests on selective medium. As shown in Fig. 2*B*, only yeast co-transformed with hα-syn and α_{1D}-C-tail were able to grow on high stringency selective medium. These findings suggest that hα-syn specifically interacts with the C terminus of α_{1D}- but not α_{1A}- or α_{1B}-ARs.

α_{1D}-AR C-tail GST Fusion Proteins Specifically Associate with Purified Syntrophin Isoforms—Five syntrophin isoforms have been cloned (α, β1, β2, γ1, γ2), each containing a highly homologous PDZ domain (23). Therefore, to examine the interaction specificity of the α_{1D}-C-tail with different syntrophin isoforms, blot overlay assays were performed using hexahistidine (His_6)/S protein-tagged syntrophin PDZ domains and α_{1D}-C-tail GST fusion proteins. The PDZ domain fusion proteins were immobilized on membranes and overlaid with GST-tagged α_{1D}-C-tail fusion proteins. As shown in Fig. 3, GST-α_{1D}-C-tail interacted robustly with α-, β1-, and β2-syntrophins and weakly associated with γ2-syntrophin. The third PDZ domain from PSD95 (PSD95-PDZ3) and the first PDZ domain from MAGI2-PDZ1 were used as negative controls. As expected, the α_{1D}-C-tail did not associate with PSD95-PDZ3 or MAGI2-PDZ1. Next, we performed a reverse blot overlay to confirm our previous findings. GST-tagged α_{1D}-C-tail fusion proteins were immobilized on membranes and overlaid with individual PDZ domain fusion proteins (Fig. 4*A*). Consistent with our previous experiment, the α_{1D}-C-tail was found to specifically interact with the PDZ domains of the α, β1, and β2 isoforms of syntrophin. In addition, the α_{1D}-C-tail did not associate with PSD95-PDZ3 and MAGI2-PDZ1, whereas consistent with previous reports (17) that the β_1-AR C terminus (β_1-C-tail) associated with both of these PDZ domains (Fig. 4*B*). Therefore, these studies suggest that the α_{1D}-C-tail selectively associates with the α, β1, and β2 isoforms of syntrophin.

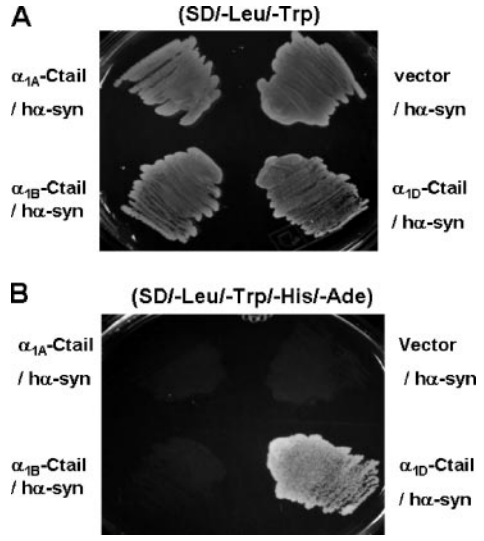

FIGURE 2. **α-Syntrophin specifically interacts with the C terminus of α_{1D}-ARs in yeast.** *A*, yeast strain AH109 co-transformed with the α-syntrophin library clone, and either the indicated receptor C-terminal constructs (α_{1A}, α_{1B}, and α_{1D}) or vector was streaked on a mating medium (SD/−Leu/−Trp). *B*, yeast streaked on *A* were restreaked on a high stringency-selective medium (SD/−Leu/−Trp/−His/−Ade).

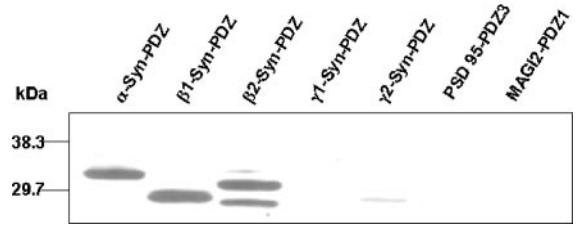

FIGURE 3. **The α_{1D}-C-tail specifically recognizes syntrophin isoforms in blot overlay assays.** 2 μg of purified His_6/S-tagged PDZ domain fusion proteins were run on SDS-PAGE, transferred to a nitrocellulose membrane, and overlaid with 25 nM GST-tagged α_{1D}-C-tail fusion protein, as described under "Experimental Procedures."

Syntrophin Isoforms Co-immunoprecipitate with α_{1D}-ARs in HEK293 Cells—Because the α-syntrophin PDZ domain was found to specifically interact with the α_{1D}-C-tail in yeast (Fig. 2*B*) and in blot overlay assays (Figs. 3 and 4), we next determined whether full-length α_{1D}-AR and syntrophins might associate in intact cells. HEK293 cells were co-transfected with α-syntrophin and a single N-terminal FLAG-tagged α_1-AR subtype. Cells were harvested, and membrane preparations were solu-

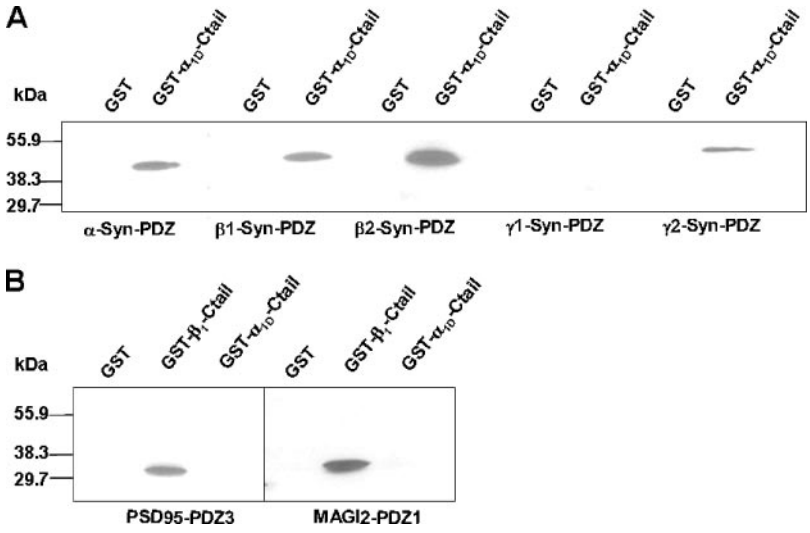

FIGURE 4. **Different syntrophin isoforms recognize the α_{1D}-C-tail in blot overlay assays.** *A*, 2 μg of purified GST proteins or GST-tagged α_{1D}-Ctail fusion proteins were run on SDS-PAGE, transferred to nitrocellulose membranes, and overlaid with 25 nM concentrations of the indicated PDZ domain fusion protein. *B*, 2 μg of purified GST proteins, GST-tagged β_1-tail, or α_{1D}-C-tail fusion proteins were run on SDS-PAGE, transferred to nitrocellulose membranes, and overlaid with 25 nM concentrations of the indicated PDZ domains.

bilized and immunoprecipitated using an anti-FLAG affinity matrix. Immunoprecipitation of receptors was resolved on a SDS-PAGE gel, and syntrophins were detected by the pan-specific syntrophin antibody that is able to recognize α, β1, and β2 syntrophins (24). As shown in Fig. 5, untransfected HEK293 cells exhibit endogenous syntrophin immunoreactivity that is somewhat enhanced by α-syntrophin transfection. Syntrophin co-immunoprecipitated with full-length α_{1D}-ARs but not with the other two α_1-AR subtypes. These data confirm that α_{1D}-ARs and syntrophins can interact in a cellular context.

The α_{1D}-AR Interacts with Syntrophins through Its C-tail in HEK293 Cells—Because the α_{1D}-AR C-tail was shown to interact with α-syntrophin in yeast, we further tested whether the receptor C-tail is the major determinant for this interaction in mammalian cells, utilizing a chimeric α_{1B}-AR with the α_{1D}-AR C-tail (FLAG-$\alpha_{1B/D}$-ARs). This C-tail chimeric receptor showed similar pharmacological properties and $G\alpha_{q/11}/Ca^{2+}$ signaling to the wild type α_{1B}-AR (data not shown). Because HEK293 cells endogenously express syntrophins (Fig. 5, *left lane*), cells were only transfected with FLAG-α_{1B}-ARs, FLAG-α_{1D}-ARs, and FLAG-$\alpha_{1B/D}$-ARs. Immunoprecipitation of FLAG-tagged receptors followed by detection for syntrophins showed that syntrophins were associated with α_{1D}-ARs and $\alpha_{1B/D}$-ARs but not with α_{1B}-ARs (Fig. 6), suggesting that the α_{1D} C-tail plays an important role in its interaction with syntrophins, probably through the PDZ-interacting motif.

Mutation of the PDZ-interacting Motif Affects α_{1D}-AR Receptor Expression and Signaling—To determine whether the α_{1D}-AR/syntrophin interaction might be involved in regulating α_{1D}-AR function, the PDZ-interacting motif at the α_{1D}-AR C-tail was substituted with alanine residues (^{568}RETDI^{572} → ^{568}AAAAA^{572}) to disrupt its interaction with syntrophins, and the function of the receptors without (HA-Ntrα_{1D}) or with the mutated PDZ-interacting motif (ΔPDZ-HA-Ntrα_{1D}) was examined by measuring accumulation of InsPs upon NE stimulation. N-terminal-truncated α_{1D}-ARs (Ntrα_{1D}-AR) were used to ensure sufficient expression, since truncation does not affect receptor pharmacological or signaling properties but dramatically increases functional receptor expression on the cell surface (11, 12). In control experiments, HA-Ntrα_{1D} was also found to co-immunoprecipitate with syntrophins when transfected in HEK293 cells (data not shown). As shown in Fig. 7, HEK293 cells stably expressing each receptor construct had similar basal InsP formations. Cells expressing HA-Ntrα_{1D} showed a sequential increase in InsP production with increasing concentrations of NE (10^{-7} and 10^{-4} M). However, those cells expressing ΔPDZ-HA-Ntrα_{1D} barely showed any increase over the basal even at 10^{-4} M NE compared with HA-Ntrα_{1D}. Both cell lines showed similar InsP formation when challenged with 10^{-4} M carbachol to stimulate endogenously expressed muscarinic cholinergic receptors, suggesting the difference in InsP formation by NE was specific to the transfected receptors.

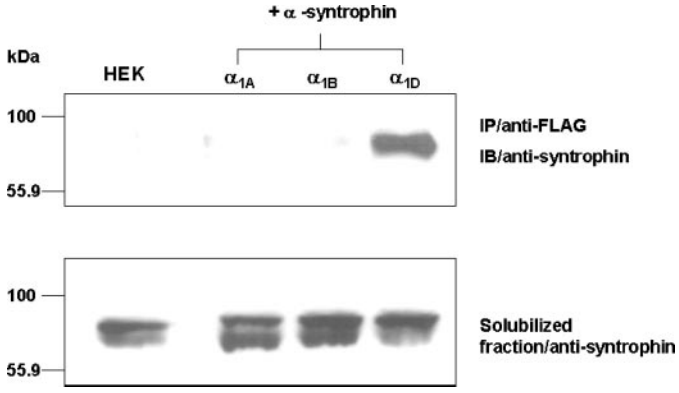

FIGURE 5. **Syntrophins interact with α_{1D}-ARs when transfected in HEK293 cells.** HEK293 cells were transfected with a FLAG-tagged α_1-AR subtype and α-syntrophin. FLAG-tagged receptors were pulled down by anti-FLAG affinity gel, and the co-immunoprecipitated (*IP*) syntrophins were detected by anti-syntrophin antibody. Aliquots of the solubilized fractions from the cell lysates were examined by Western blotting (*IB*) to visualize syntrophin expression.

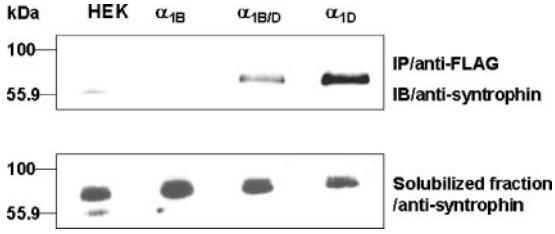

FIGURE 6. **Syntrophins specifically interact with the α_{1D}-AR C terminus in HEK293 cells.** HEK293 cells were transfected with FLAG-tagged α_{1B}-AR, chimeric α_{1B} with the C terminus of the α_{1D}-AR ($\alpha_{1B/D}$), or α_{1D}-AR cDNAs. FLAG-tagged receptors were pulled down by anti-FLAG affinity gel, and the co-immunoprecipitated (*IP*) syntrophins were detected by anti-syntrophin antibody. Aliquots of the solubilized fractions from cell lysates were examined by Western blotting (*IB*) to visualize syntrophin expression.

To test whether this dramatic decrease in signaling is caused by a difference in receptor expression, saturation binding assays with the α_1-AR specific radioligand ^{125}I-labeled BE were performed (Fig. 8*A*). The receptor densities were 1916 ± 41 fmol/mg of protein for cells expressing HA-Ntrα_{1D} and 301 ± 29 fmol/mg for those with ΔPDZ-

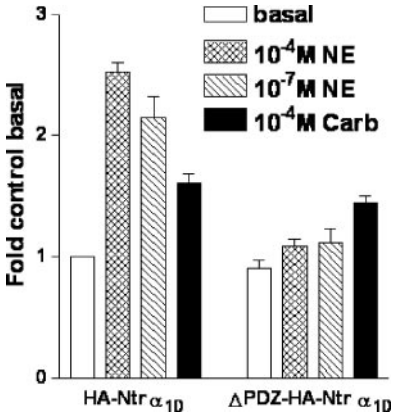

FIGURE 7. **NE-stimulated ^{3}H-labeled InsP formation in HEK293 cells expressing the indicated HA-tagged α_{1D}-AR constructs.** ^{3}H-Labeled InsP formation by different concentrations of NE and 10^{-4} M carbachol (*Carb*) was measured in HEK293 cells transfected with N-terminal-truncated α_{1D}-AR construct (*left*, HA-Ntrα_{1D}) or N-terminal-truncated α_{1D}-AR construct with a mutated PDZ-interacting motif (*right*, ΔPDZ-HA-Ntrα_{1D}). The data were normalized to the basal level of cells expressing HA-Ntrα_{1D} and plotted as -fold control basal. *Bars* represent the mean ± S.E. of six measurements for each condition.

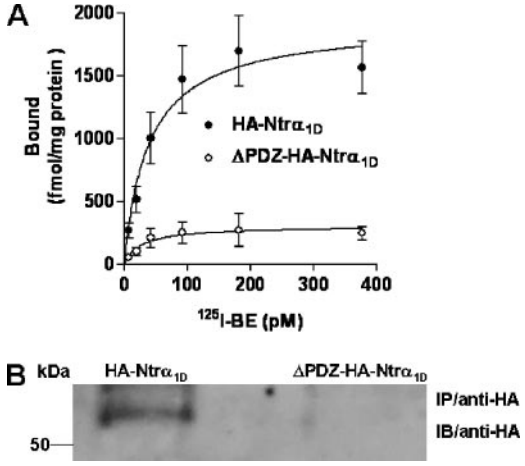

FIGURE 8. **Mutation of the PDZ-interacting motif at the α_{1D}-AR C-tail affects receptor levels.** *A*, saturation binding of ^{125}I-labeled BE to HEK293 membranes expressing HA-Ntrα_{1D} (●) or ΔPDZ-HA-Ntrα_{1D} (○). *Symbols* represent the mean ± S.E. of three experiments performed in duplicate. *B*, immunoprecipitation (*IP*) and Western blotting (*IB*) of protein expression of HA-Ntrα_{1D} and ΔPDZ-HA-Ntrα_{1D} stably expressed in HEK293 cells.

HA-Ntrα_{1D}. Because the heterologously expressed α_{1D}-AR has been found to show a dramatic discrepancy between protein expression level and receptor density, probably due to its intracellular localization (14, 16), protein expression of those two α_{1D} constructs was examined by immunoprecipitation and Western blotting (Fig. 8*B*). ΔPDZ-HA-Ntrα_{1D} was expressed at a much lower level (nearly undetectable) compared with HA-Ntrα_{1D}, suggesting that the poor receptor density was caused by impaired protein expression.

Overexpression of α-Syntrophin Increases Receptor Protein and Cell Surface Expression—Because our previous data showed that mutation of the PDZ-interacting motif at the receptor C-tail affects receptor protein expression, we wanted to determine whether syntrophins were involved. As shown in Fig. 9*A*, when α-syntrophin was stably overexpressed in HEK293 cells that were then transiently transfected with either the HF-α_{1D}-AR or the ΔPDZ-HF-Ntrα_{1D} mutant, increased α_{1D}-AR protein expression (2.3 ± 0.6-fold higher; $n = 3$) was observed by Western blotting. However, the expression of the ΔPDZ-HF-Ntrα_{1D} mutant was not significantly altered by stable α-syntrophin overexpression.

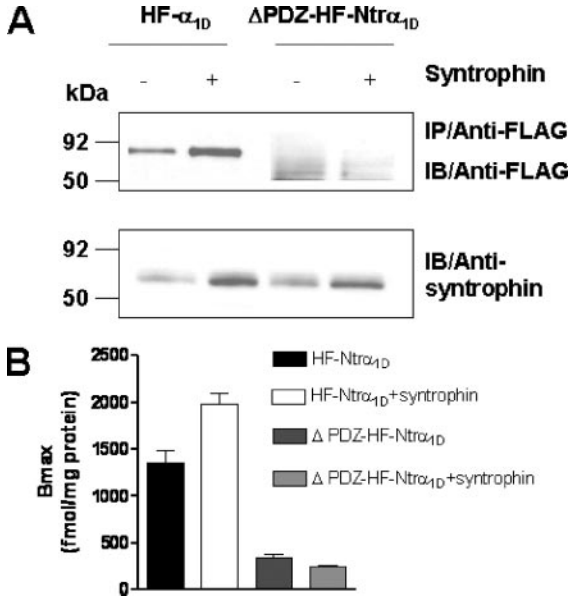

FIGURE 9. **Overexpression of α-syntrophin induces an increase in receptor protein expression and binding.** *A*, immunoprecipitation (*IP*) and Western blotting (*IB*) of protein expression of HF-α1D and ΔPDZ-HF-Ntrα1D transiently expressed in HEK293 cells stably overexpressing α-syntrophin. *B*, *bar graph* of the B_{max} of 125I-BE binding to HEK293 membranes expressing HF-Ntrα1D or ΔPDZ-HF-Ntrα1D alone or along with overexpressed α-syntrophin.

To test whether this increase in protein expression might result in an increase in functional receptors, saturation binding assays with ^{125}I-labeled BE were performed on cell membranes (Fig. 9*B*). Receptor densities were 1353 ± 271 fmol/mg of protein for cells expressing HF-Ntrα_{1D} alone and 1977 ± 229 fmol/mg ($n = 4$; $p < 0.02$) for cells also overexpressing α-syntrophin, indicative of an increase in cell surface expression. However, there was no change in ΔPDZ-HF-Ntrα_{1D} expression in the presence or absence of overexpressed α-syntrophin. This suggests that α-syntrophin plays an important role in regulation of α_{1D}-AR protein expression and/or stability by a direct interaction with the PDZ-interacting motif at the α_{1D}-AR C-tail.

DISCUSSION

In this study α-syntrophin was identified as a novel interacting protein for the α_{1D}-AR in a yeast two-hybrid screen. Comparison of the C-terminal amino acids (RETDI) of the α_{1D}-AR with the ideal syntrophin PDZ-interacting motif ((K/Q/R)E(S/T)*X*(V/I)) (20–22) showed a remarkable degree of identity. Studies using biochemical assays and co-immunoprecipitation in mammalian cells further determined the interaction specificity between the five syntrophin isoforms (α, β1, β2, γ1 and γ2) and the three α_1-AR subtypes (α_{1A}, α_{1B} and α_{1D}). The data indicated that the α_{1D}-AR strongly interacts with three syntrophin isoforms (α, β1, and β2) via its C-terminal domain and that syntrophins specifically associate with α_{1D}-ARs but not the other α_1-AR subtypes. Mutation of the PDZ-interacting motif at the α_{1D}-AR C terminus caused a dramatic decrease in receptor protein expression, binding, and signaling. Overexpression of α-syntrophin did not rescue this decrease but did increase both protein expression and binding of the receptor with the intact PDZ interacting motif.

Syntrophins, like many PDZ domain-containing proteins, have been shown to play a key role in anchoring proteins to the cell membrane for properly assembling various signaling complexes, such as neuronal nitric-oxide synthase (25, 26), voltage-gated sodium channels (20, 27), water channel protein aquaporins-4 (26, 28), stress-activated protein kinase-3 (29), and Grb 2 (30). Five mammalian syntrophin isoforms (α,

β1, β2, γ1, and γ2) have been cloned, each containing at least one pleckstrin homology (PH) domain, a conserved PDZ domain, and a C-terminal syntrophin-unique domain responsible for interaction with dystrophin (31). PH domains are known to bind to phosphatidylinositol 4,5-bisphosphate, a key component in $G\alpha_{q/11}$ signaling (32). This pathway is the primary signaling mechanism for α_1-ARs (1). However, the function of PH domains in syntrophins is currently unknown. Although PH domain 1 of α-syntrophin has been shown to bind to phosphatidylinositol 4,5-bisphosphate at a biochemical level (33), syntrophins have not yet been directly implicated in G protein-coupled receptor signaling or other phosphatidylinositol 4,5-bisphosphate interactions. Therefore, the finding that syntrophins directly and specifically associate with α_{1D}-ARs may provide new insights into their functions.

Although previous studies based on protein sequence alignment showed that the five syntrophin isoforms contain PDZ domains with high homologies (23), our findings and other studies suggest that these domains show slightly different specificities in recognizing PDZ-interacting motifs. α, β2, and γ1 syntrophins are able to interact with phosphoinositol 3,4-bisphosphate-binding protein TAPP1 (34), whereas only α, β1, β2 syntrophins showed consistent interactions with α_{1D}-ARs in our experiments, suggesting that α and β2 syntrophins may share similarity in terms of recognition of PDZ-interacting motifs. This idea has been supported by a recent study on α/β2-syntrophin null mice, where α and β2 syntrophins were found to be able to compensate for the functions of the other isoform (35).

In the present study mutation of the PDZ-interacting motif of the α_{1D}-AR resulted in greatly impaired receptor protein expression and signaling, suggesting the PDZ-interacting motif may determine receptor expression and/or stability. Besides directing the appropriate cell surface targeting of many proteins via PDZ domain-mediated interactions, syntrophins are also known to regulate the stability of other proteins. Deletion of only three amino acids in the PDZ-interacting motif of aquaporin-4 increased the protein degradation rate from a half-life of 24 to 8 h (28). We found that overexpression of α-syntrophin significantly increased α_{1D}-AR expression but not that with a mutated PDZ-interacting motif, which is consistent with many previous reports demonstrating that syntrophins increase the stability of various cellular proteins (28, 36, 37). In addition, β2-syntrophin was found to stabilize islet cell autoantigen 512 (ICA512) by binding to its C-terminal PDZ-interacting motif, thus masking the nearby PEST sequence (38) and preventing the cleavage of ICA512 by calpain (36). Interestingly, α_{1D}-ARs also contain a putative PEST sequence between amino acids Arg^{446} and Ser^{487} (score, +8.22), suggesting that disruption of the interaction between syntrophins and α_{1D}-ARs by mutating the PDZ-interacting motif may expose the PEST sequence to proteolytic attack, thereby accounting for the dramatic decrease in receptor levels observed in this study.

α-ARs play an important role in vasoconstriction mediated by NE. Because syntrophins interact with α_{1D}-ARs, they might modulate receptor expression and may, therefore, affect α_{1D}-AR-mediated vasoconstriction in syntrophin null mice. However, NE-mediated vasoconstriction in hind limb is not altered in α-syntrophin null mice (39). This could be explained by the presence of other compensatory syntrophin isoforms (35), since at least three isoforms associate with α_{1D}-ARs. Alternatively, this result could be due to the lack of involvement of α_{1D}-ARs in hindlimb vasoconstriction, since the specific α_1-AR subtypes involved in this phenomenon are currently unknown. To determine whether syntrophins affect α_{1D}-AR expression and/or function would require further characterization of α_{1D}-AR-mediated vasoconstriction in mice lacking multiple syntrophin isoforms.

Another interesting finding in our set of data reported here is that syntrophins seem to interact equally well with α_{1D}-ARs mainly expressed intracellularly (full-length) (10, 11) and α_{1D}-ARs expressed at the cell surface (N-terminal-truncated receptors) (11, 12), suggesting that syntrophins may function in different subcellular compartments. Although syntrophins have been thought to function mainly at the cell surface as a component in the dystrophin-associated glycoprotein complex (31), a recent study shows that α, β1, and β2 syntrophins were found to localize at the cell surface and also in the cytosolic fraction in cardiac muscle (40), suggesting that syntrophins may function independent of association with dystrophins. Because α-syntrophin has been found to modulate protein stability, the interaction between syntrophins and α_{1D}-ARs may partly account for the puzzling observation that in heterologously expressed cells α_{1D}-ARs accumulate intracellularly at a very high level without being degraded (16).

The data presented in this study clearly show that α_{1D}-ARs specifically and strongly interact via their C termini with the PDZ domains of syntrophin isoforms (α, β1, and β2). This idea is supported by the presence of the well defined syntrophin PDZ-interacting consensus sequence in the last five amino acids of the α_{1D}-AR C terminus, suggesting that this interaction occurs with a high affinity *in vivo*. To our knowledge this is the first report of syntrophins interacting with G protein-coupled receptors. In addition, it is interesting that this particular receptor utilizes phosphatidylinositol 4,5-bisphosphate as its major signaling component, providing a potential function for the syntrophin PH domain(s). This novel interaction between syntrophins and α_{1D}-ARs may play an important role in receptor stability, since mutation of the α_{1D}-AR PDZ-interacting motif caused a dramatic decrease in receptor protein and function, and overexpression of α-syntrophin caused an increase in receptor expression. Because this interaction is unique to the α_{1D}-AR, further investigation of the functional role of this interaction may broaden our knowledge of the differences between the three α_1-AR subtypes.

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