Syntrophins Regulate $\alpha_{1D}$-Adrenergic Receptors through a PDZ-Domain-Mediated Interaction*

Zhongjian Chen, Chris Hague, Randy A. Hall, and Kenneth P. Minneman

From the Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

To find novel cytoplasmic binding partners of the $\alpha_{1D}$-adrenergic receptor (AR), a yeast two-hybrid screen using the $\alpha_{1D}$-AR C terminus as bait was performed on a human brain cDNA library. $\alpha$-Syntrophin, a protein containing one PDZ domain and two pleckstrin homology domains, was isolated in this screen as an $\alpha_{1D}$-AR-interacting protein. $\alpha$-Syntrophin specifically recognized the C terminus of $\alpha_{1D}$-AR but not $\alpha_{1A}$- or $\alpha_{1B}$-ARs. In blot overlay assays, the PDZ domains of syntrophin isoforms $\alpha_1$, $\beta_1$, and $\beta_2$ but not $\gamma_1$ or $\gamma_2$ showed strong selective interactions with the $\alpha_{1D}$-AR C-tail fusion protein. In transfected human embryonic kidney 293 cells, full-length $\alpha_{1D}$-AR but not $\alpha_{1A}$- or $\alpha_{1B}$-ARs co-immunoprecipitated with syntrophin, and the importance of the receptor C terminus for the $\alpha_{1D}$-AR/syntrophin interaction was confirmed using chimeric receptors. Mutation of the PDZ-interacting motif at the $\alpha_{1D}$-AR C terminus markedly decreased inositol phosphate formation stimulated by norepinephrine but not carbachol in transfected HEK293 cells. This mutation also dramatically decreased $\alpha_{1D}$-AR binding and protein expression. In addition, stable overexpression of $\alpha$-syntrophin significantly increased $\alpha_{1D}$-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 $\alpha_{1D}$-AR signaling and the role of syntrophins in modulating G protein-coupled receptor function.

$\alpha_1$-Adrenergic receptors ($\alpha_1$-ARs) 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 $\alpha_1$-AR subtypes have been cloned ($\alpha_1A$, $\alpha_1B$, and $\alpha_1D$-$AR$) and display differences in sequence homology and affinities for subtype selective ligands (1). Upon agonist stimulation, all three $\alpha_1$-AR subtypes signal through $G_{q/11}$ to increase phospholipase C activity and intracellular $\text{Ca}^{2+}$ mobilization (1, 2). In addition, recent studies using $\alpha_1$-AR transgenic and knock-out mice have now revealed that all three $\alpha_1$-AR subtypes are important for the regulation of blood pressure (2, 3). Therefore, it remains unclear if specific functional differences exist between the $\alpha_1$-AR subtypes.

Increasing evidence now suggests that $\alpha_1$-AR subtypes display differences in their ability to interact with specific protein binding partners. The first $\alpha_1$-AR subtype-selective binding partner identified was tissue transglutaminase II, which selectively associates with $\alpha_1B$- and $\alpha_1D$-ARs (4, 5). Since then, other proteins found to selectively associate with $\alpha_1$-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 $\alpha_1$-ARs. Therefore, these differential interactions may contribute to subtype-specific differences between the members of the $\alpha_1$-AR family.

Previously, the $\alpha_{1D}$-AR was the least studied of the $\alpha_1$-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 $\alpha_{1B}$-ARs (13, 14) or $\beta_2$-ARs (15) promotes $\alpha_{1D}$-AR cell surface expression and increases coupling to functional responses. However, it remains unknown if there are any $\alpha_{1D}$-AR accessory proteins involved in $\alpha_{1D}$-AR function and/or expression.

In this study, we have identified several closely related syntrophin family isoforms ($\alpha_1$, $\beta_1$, and $\beta_2$) as novel $\alpha_1$-AR binding partners using a combination of yeast two-hybrid screening and biochemical techniques. We report that syntrophins directly interact with $\alpha_{1D}$-ARs through a PDZ domain-mediated interaction. The specificity of this association and its potential role in regulating $\alpha_{1D}$-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 (Qiogene, 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% FBS (Hyclone, Logan, UT), dihydrotestosterone (Sigma-Aldrich). Cell culture plates: 24-well plates (Costar, Cambridge, MA). Antibodies: mouse anti-syntrophins antibody (MA1–745, Affinity BioReagents, Golden, CO).

**Yeast Two-Hybrid Screening**—Plasmid pGBK7/α1D-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...
interaction at the PDZ-interacting motif. The chimeric resultant from positive colonies by transforming yeast DNA extracts into buffer P1 by vortexing for 5 min. Library plasmid DNA was rescued AGCAGCAGCAGCTGCTTAAACGCGTGCT (reverse), digested with (mouse human mutated constructs were swapped by EcoRI and MluI to generate the f o r1 ha troom temperature (RT), subsequently incubated with 25 nM saline containing 0.1% Tween 20 (TBST) consisting of 5% nonfat milk for 1-2 h at room temperature (RT), and then washed with 1× buffer (25 mM HEPES, 150 mM NaCl, pH 7.4) with a protease inhibitor mixture (1 mM benzamidine, 3 mM pepstatin, 3 mM phenylmethylsulfonyl fluoride, 3 mM 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, 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× dodecyl-D-maltoside, and the supernatant was incubated with 4× dodecyl-D-maltoside prepared in 1× 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 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. 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 α1D-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 His6/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 105 units/liter penicillin at 37 °C in a humidified atmosphere with 5% CO2. 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 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-maltose 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× Lae mml sample buffer (62.5 mM Tris-HCl, pH 6.8, 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-α1D-AR and ΔPDZ-HF-Ntr1D-AR, immunoprecipitation was done as previously described (19).

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

3H-Labeled Inositol Phosphate (InsP) Formation—Receptor function in transfected HEK293 cells was measured by [3H]inositol phosphate (InsP) formation upon NE stimulation as previously described (18). 10−8 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, Kd and B max were calculated by nonlinear regression using Prism (GraphPad).
Interaction of Syntrophins with α_{1D}-ARs

RESULTS

α-Syntrophin Is a Specific Binding Partner for the α_{1D}-AR C Terminal—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 (ho-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 (Consensus PDZ-interacting motif) 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 ho-syn and α_{1D}-C-tail was confirmed by further yeast two-hybrid analysis. The ho-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 ho-syn (Fig. 2A) were subjected to growth tests on selective medium. As shown in Fig. 2B, only yeast co-transformed with ho-syn and α_{1D}-C-tail were able to grow on high stringency selective medium. These findings suggest that ho-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 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. 4A). 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 β1-AR C terminus (β1-C-tail) associated with both of these PDZ domains (Fig. 4B). Therefore, these studies suggest that the α_{1D}-C-tail selectively associates with the α, β1, and β2 isoforms of syntrophin.

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. 2B) 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-
Interaction of Syntrophins with α1D-ARs

FIGURE 4. Different syntrophin isoforms recognize the α1D-C-tail in blot overlay assays. A, 2 μg of purified GST proteins or GST-tagged α1D/C-tail 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 β1-tail, or α1D/C-tail fusion proteins were run on SDS-PAGE, transferred to nitrocellulose membranes, and overlaid with 25 nM concentrations of the indicated PDZ domains.

FIGURE 5. Syntrophins interact with α1D-ARs when transfected in HEK293 cells. HEK293 cells were transfected with a flag-tagged α1D sub-type 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 α1D-AR C terminus in HEK293 cells. HEK293 cells were transfected with a flag-tagged α1D-AR, chimeric α1D with the C terminus of the α1D-AR (α1D-HA), or α1D-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.

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 α1D-ARs but not with the other two α1-AR subtypes. These data confirm that α1D-ARs and syntrophins can interact in a cellular context.

The α1D-AR Interacts with Syntrophins through Its C-tail in HEK293 Cells—Because the α1D-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 α1D-AR with the α1D-AR C-tail (FLAG-α1D-AR). This C-tail chimeric receptor showed similar pharmacological properties and Ga2z/Ca2+ signaling to the wild type α1B-AR (data not shown). Because HEK293 cells endogenously express syntrophins (Fig. 5, left lane), cells were only transfected with FLAG-α1D-ARs, FLAG-α1D-ARs, and FLAG-α1B/1D-ARs. Immunoprecipitation of FLAG-tagged receptors followed by detection for syntrophins showed that syntrophins were associated with α1D-ARs and α1B/1D-ARs but not with α1B-ARs (Fig. 6), suggesting that the α1D-AR C-tail plays an important role in its interaction with syntrophins, probably through the PDZ-interacting motif.

Mutation of the PDZ-interacting Motif Affects α1D-AR Receptor Expression and Signaling—To determine whether the α1D-AR/syntrophin interaction might be involved in regulating α1D-AR function, the PDZ-interacting motif at the α1D-AR C-tail was substituted with alanine residues (568RETDA572→568AAAA572) to disrupt its interaction with syntrophins, and the function of the receptors without (HANtrα1D) or with the mutated PDZ-interacting motif (ΔPDZ-HA-Ntrα1D) was examined by measuring accumulation of InsPs upon NE stimulation. N-terminal-truncated α1D-ARs (Ntrα1D) 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-Ntrα1D 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-Ntrα1D showed a sequential increase in InsP production with increasing concentrations of NE (10−7 and 10−4 M). However, those cells expressing ΔPDZ-HA-Ntrα1D barely showed any increase over the basal even at 10−4 M NE compared with HA-Ntrα1D. Both cell lines showed similar InsP formation when challenged with 10−3 M carbachol to stimulate endogenously expressed muscarinic cholinergic receptors, suggesting the difference in InsP formation by NE was specific to the transfected receptors.

To test whether this dramatic decrease in signaling is caused by a difference in receptor expression, saturation binding assays with the α1-AR specific radioligand 125I-labeled BE were performed (Fig. 8A). The receptor densities were 1916 ± 41 fmol/mg for cells expressing HA-Ntrα1D and 301 ± 29 fmol/mg for those with ΔPDZ-
Interaction of Syntrophins with α₁D-ARs

FIGURE 7. NE-stimulated [³H]-labeled InsP₃ formation in HEK293 cells expressing the indicated HA-tagged α₁D-AR constructs. [³H]-Labeled InsP₃ formation by different concentrations of NE and 10⁻⁴ M carbachol (Carb) was measured in HEK293 cells transfected with N-terminal-truncated α₁D-AR construct (left, HA-Ntrα₁D) or N-terminal-truncated α₁D-AR construct with a mutated PDZ-interacting motif (right, ΔPDZ-HA-Ntrα₁D). The data were normalized to the basal level of cells expressing HA-Ntrα₁D, 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 α₁D-AR C-tail affects receptor levels. A, saturation binding of [¹²⁵]I-labeled BE to HEK293 membranes expressing HA-Ntrα₁D (●) or ΔPDZ-HA-Ntrα₁D (○). Symbols represent the mean ± S.E. of three experiments performed in duplicate. B, immunoprecipitation (IP) and Western blotting (IB) of protein expression of HA-Ntrα₁D and ΔPDZ-HA-Ntrα₁D stably expressed in HEK293 cells.

HA-Ntrα₁D. Because the heterologously expressed α₁D-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 α₁D constructs was examined by immunoprecipitation and Western blotting (Fig. 8B). ΔPDZ-HA-Ntrα₁D was expressed at a much lower level (nearly undetectable) compared with HA-Ntrα₁D, 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. 9A, when α-syntrophin was stably overexpressed in HEK293 cells that were then transiently transfected with either the HF-α₁D-AR or the ΔPDZ-HF-Ntrα₁D mutant, increased α₁D-AR protein expression (2.3 ± 0.6-fold higher; n = 3) was observed by Western blotting. However, the expression of the ΔPDZ-HF-Ntrα₁D mutant was not significantly altered by stable α-syntrophin overexpression.

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. 9B). Receptor densities were 1535 ± 271 fmol/mg of protein for cells expressing HF-Ntrα₁D 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-Ntrα₁D expression in the presence or absence of overexpressed α-syntrophin. This suggests that α-syntrophin plays an important role in regulation of α₁D-AR protein expression and/or stability by a direct interaction with the PDZ-interacting motif at the α₁D-AR C-tail.

DISCUSSION

In this study α-syntrophin was identified as a novel interacting protein for the α₁D-AR in a yeast two-hybrid screen. Comparison of the C-terminal amino acids (RETDL) of the α₁D-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 (α₁, α₂, α₃, α₄, and α₅) and the three α₁D-AR subtypes (α₁D, α₁D₂, and α₁D₃). The data indicated that the α₁D-AR strongly interacts with three syntrophin isoforms (α₁, α₂, and α₃) via its C-terminal domain and that syntrophins specifically associate with α₁D-ARs but not the other α₁D-AR subtypes. Mutation of the PDZ-interacting motif at the α₁D-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 (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 Gβγ11 signaling (32). This pathway is the primary signaling mechanism for α1-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 α1D-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 slight differences in recognizing PDZ-interacting motifs. α, β2, and γ1 syntrophins are able to interact with phosphoinositols 3,4-bisphosphate-binding protein TAPP1 (34), whereas only α, β1, β2 syntrophins showed consistent interactions with α1D-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 α1D-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 α1D-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, the C-terminal syntrophin-unique domain responsible for interaction with dystrophin (31). PH domains are known to bind to phosphatidylinositol 4,5-bisphosphate 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 α1D-ARs may partly account for the puzzling observation that in heterologously expressed cells α1D-ARs accumulate intracellularly at a very high level without being degraded (16).

The data presented in this study clearly show that α1D-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 α1D-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 α1D-ARs may play an important role in receptor stability, since mutation of the α1D-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 α1D-AR, further investigation of the functional role of this interaction may broaden our knowledge of the differences between the three α1-AR subtypes.

Acknowledgments—We thank Amanda Castleberry and Sarah Lee for outstanding technical assistance.

REFERENCES
1. Zhong, H., and Minneman, K. P. (1999) Eur. J. Pharmacol. 375, 261–276
2. Hague, C., Cheri, Z., Uberti, M., and Minneman, K. P. (2003) J. Pharmacol. Exp. Ther. 307, 1008–1016
3. Tanour, A., Koshimizu, T. A., and Tsujimoto, G. (2002) Eur. J. Pharmacol. 411–418
4. Uberti, M. A., Hague, C., Oller, H., Minneman, K. P., and Hall, R. A. (2005) J. Biol. Chem. 280, 1379–1390
5. Chen, S., Lin, F., Iismaa, S., Lee, K. N., Birckbichler, P. J., and Graham, R. M. (1994) Science 264, 1593–1596
6. Wang, X., Zeng, W., Soyombo, A. A., Tang, W., Ross, E. M., Barnes, A. P., Milgram, S. L., Pembinger, J. M., Allen, P. B., Greengard, P., and Muallem, S. (2005) Nat. Cell Biol. 7, 405–411
7. Diviani, D., Lattion, A. L., Abouin, L., Staehl, O., and Cotechica, S. (2003) J. Biol. Chem. 278, 19331–19340
8. Hague, C., Bernstein, L. S., Ramineni, S., Chen, Z., Minneman, K. P., and Hepler, J. R. (2005) J. Biol. Chem. 280, 27279–27291
9. Wang, X., Zeng, W., Soyombo, A. A., Tang, W., Ross, E. M., Barnes, A. P., Miligam, S. L., Pembinger, J. M., Allen, P. B., Greengard, P., and Muallem, S. (2005) Nat. Cell Biol. 7, 405–411
10. Chalothorn, D., McCune, D. F., Edelmann, S. E., Garcia-Cazorin, M. L., Tsujimoto, G., and Piscik, M. T. (2002) Mol. Pharmacol. 61, 1008–1016
11. Hague, C., Cheri, Z., Uberti, M., Schulte, N., Toews, M. L., and Minneman, K. P. (2004) J. Pharmacol. Exp. Ther. 309, 388–397
12. Uberti, M. A., Hague, C., Hall, R. A., and Minneman, K. P. (2003) Mol. Pharmacol. 64, 1379–1390
13. Hague, C., Uberti, M. A., Chen, Z., Hall, R. A., and Minneman, K. P. (2004) J. Biol. Chem. 279, 15541–15549
14. Uberti, M. A., Hague, C., Oller, H., Minneman, K. P., and Hall, R. A. (2005) J. Pharmacol. Exp. Ther. 313, 16–23
15. Vicentini, A., Robeva, A., Rogge, G., Uberti, M., and Minneman, K. P. (2002) J. Pharm.
Interaction of Syntrophins with α_{1D}-ARs

17. Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001) J. Biol. Chem. 276, 41310–41317
18. Chen, Z., Rogge, G., Hague, C., Alewood, D., Colless, B., Lewis, R. J., and Minneman, K. P. (2004) J. Biol. Chem. 279, 35326–35333
19. Balasubramanian, S., Teissere, J. A., Raju, D. V., and Hall, R. A. (2004) J. Biol. Chem. 279, 18840–18850
20. Gee, S. H., Madhavan, R., Levinson, S. R., Caldwell, J. H., Sealock, R., and Froehner, S. C. (1998) J. Neurosci. 18, 128–137
21. Gee, S. H., Quenneville, S., Lombardo, C. R., and Chabot, J. (2000) Biochemistry 39, 14638–14646
22. Wiedemann, U., Boisguerin, P., Leben, R., Leitner, D., Krause, G., Moelling, K., Vollmer-Engert, R., and Oschkinat, H. (2004) J. Mol. Biol. 343, 703–718
23. Piluso, G., Mirabella, M., Ricci, E., Belsito, A., Abbondanza, C., Servidei, S., Puca, A. A., Tonali, P., Puca, G. A., and Nigro, V. (2000) J. Biol. Chem. 275, 15851–15860
24. Peters, M. F., Adams, M. E., and Froehner, S. C. (1997) J. Cell Biol. 138, 81–93
25. Brennan, J. E., Chao, D. S., Gee, S. H., McGuire, A. W., Craven, S. E., Santillano, D. R., Wu, Z., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996) Cell 84, 757–767
26. Adams, M. E., Mueller, H. A., and Froehner, S. C. (2001) J. Cell Biol. 155, 113–122
27. Schultz, J., Hoffmuller, U., Krause, G., Ashurst, J., Macias, M. I., Schmieder, P., Schneider-Mergener, J., and Oschkinat, H. (1998) Nat. Struct. Biol. 5, 19–24
28. Neely, J. D., Amiry-Moghaddam, M., Ottersen, O. P., Froehner, S. C., Agre, P., and Adams, M. E. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 14108–14113
29. Hasegawa, M., Cuenda, A., Spillantini, M. G., Thomas, G. M., Buee-Scherrer, V., Cohen, P., and Goedert, M. (1999) J. Biol. Chem. 274, 12626–12631
30. Oak, S. A., Russo, K., Petrucci, T. C., and Jarrett, H. W. (2001) Biochemistry 40, 11270–11278
31. Albrecht, D. E., and Froehner, S. C. (2002) Neurosignals 11, 123–129
32. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) Nature 371, 168–170
33. Chockalingam, P. S., Gee, S. H., and Jarrett, H. W. (1999) Biochemistry 38, 5596–5602
34. Hogan, A., Yakubchyk, Y., Chabot, J., Obagi, C., Daher, E., Maekawa, K., and Gee, S. H. (2004) J. Biol. Chem. 279, 53717–53724
35. Adams, M. E., Kramarcy, N., Fukuda, T., Engel, A. G., Sealock, R., and Froehner, S. C. (2004) J. Neurosci. 24, 10302–10309
36. Ort, T., Voronov, S., Gao, J., Zawalich, K., Froehner, S. C., Zawalich, W., and Solimena, M. (2001) EMBO J. 20, 4013–4023
37. Munehira, Y., Ohnishi, T., Kawamoto, S., Furuya, A., Shitara, K., Imamura, M., Yokota, T., Takeda, S., Amachi, T., Matsu, M., Kioka, N., and Ueda, K. (2004) J. Biol. Chem. 279, 15091–15095
38. Rechsteiner, M., and Rogers, S. W. (1996) Trends Biochem. Sci. 21, 267–271
39. Thomas, G. D., Shaul, P. W., Yuhanna, I. S., Froehner, S. C., and Adams, M. E. (2003) Circ. Res. 92, 554–560
40. Iwata, Y., Shigekawa, M., and Wakabayashi, S. (2005) Mol. Cell. Biochem. 268, 59–66