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Blood Pressure Is Regulated by an α_{1D}-Adrenergic Receptor/Dystrophin Signalosome*

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Hypertension is a cardiovascular disease associated with increased plasma catecholamines, overactivation of the sympathetic nervous system, and increased vascular tone and total peripheral resistance. A key regulator of sympathetic nervous system function is the α_{1D}-adrenergic receptor (AR), which belongs to the adrenergic family of G-protein-coupled receptors (GPCRs). Endogenous catecholamines norepinephrine and epinephrine activate α_{1D}-ARs on vascular smooth muscle to stimulate vasoconstriction, which increases total peripheral resistance and mean arterial pressure. Indeed, α_{1D}-AR KO mice display a hypotensive phenotype and are resistant to salt-induced hypertension. Unfortunately, little information exists about how this important GPCR functions because of an inability to obtain functional expression *in vitro*. Here, we identified the dystrophin proteins, syntrophin, dystrobrevin, and utrophin as essential GPCR-interacting proteins for α_{1D}-ARs. We found that dystrophins complex with α_{1D}-AR both *in vitro* and *in vivo* to ensure proper functional expression. More importantly, we demonstrate that knock-out of multiple syntrophin isoforms results in the complete loss of α_{1D}-AR function in mouse aortic smooth muscle cells and abrogation of α_{1D}-AR-mediated increases in blood pressure. Our findings demonstrate that syntrophin and utrophin associate with α_{1D}-ARs to create a functional signalosome, which is essential for α_{1D}-AR regulation of vascular tone and blood pressure.

The α_1-adrenergic receptors (AR)[2] are Class A G-protein-coupled receptors (GPCRs) that are important clinical targets for the treatment of cardiovascular disease and benign prostatic hypertrophy. Each α_1-AR subtype (α_{1A}, α_{1B}, and α_{1D}) signals through $G\alpha_{q/11}$, activates phospholipase $C\beta$ (PLCβ), and increases intracellular [Ca^{2+}] (1, 2). Despite ubiquitous expression, α_1-ARs are best characterized for their role in the cardiovascular system, where studies using α_1-AR knock-out (KO) have revealed a critical role in the regulation of blood pressure and cardiac function (3–6). The role of α_1-ARs in the central nervous system is less clear, although expression in the brain has been implicated in regulating pyschostimulant effects of drugs of abuse, learning, and memory (2, 7). The recent discovery that prazosin, an α_1-AR-selective antagonist, is an effective treatment for reoccurring nightmares in Iraqi Freedom combat veterans suffering from post-traumatic stress disorder (8, 9) emphasizes the need to understand the basic pharmacological and molecular characteristics of this important class of GPCRs.

Information on the α_{1D}-AR subtype is scant because of difficulties in heterologous expression. α_{1D}-AR cDNA expressed *in vitro* results in protein expression lacking α_{1D}-AR-binding sites and signaling responses (10, 11). It is increasingly recognized that most GPCRs are not functionally expressed in heterologous cell systems, suggesting that most GPCRs require other factors for functional expression *in vitro*. We propose that the difficulties in studying α_{1D}-AR *in vitro* stem from an absence of critical α_{1D}-AR-interacting proteins that are necessary for proper folding, expression, trafficking, localization, and signaling.

It is now appreciated that most GPCRs exist as multi-protein complexes comprised of varying numbers of GPCR-interacting proteins (GIPs), capable of regulating GPCR signaling, ligand binding, trafficking, or scaffolding to effector molecules (12). A number of α_1-AR GIPs have been identified, including RGS2 and snapin for α_{1A}-AR (13, 14) and adaptor protein complex 2, ezrin, spinophilin, and gC1qR for α_{1B}-AR (15–19). However, *bona fide* α_{1D}-AR GIPs remain elusive.

Recently, we identified syntrophins as potential α_{1D}-AR GIPs through a yeast two-hybrid screen (20). Syntrophins are important scaffolds in the dystrophin-associated complex, regulating the spatial and temporal organization of a number of signal transduction proteins (nNOS, Aquaporin 4, plasma membrane calcium ATPase1/4, stress-activated protein kinase 3, and Na_v ion channels) (21–25). The five isoforms of syntrophins (α, β_1, β_2, γ_1, and γ_2) display conserved structural features, including two pleckstrin homology (PH) domains, a PSD-95/DlgA/Zo-1 (PDZ) domain, and a syntrophin unique (SU) domain (26, 27). Given that the α_{1D}-AR interacts with syntrophins (20), we hypothesized that syntrophins may be the missing requirement for α_{1D}-AR functional expression *in vitro*.

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[2] The abbreviations used are: AR, adrenergic receptor; GPCR, G-protein-coupled receptor; GIP, GPCR-interacting protein; PLC, phospholipase C; KO, knock-out; PMCA, plasma membrane calcium ATPase; PH, pleckstrin homology; SU, syntrophin unique; GFP, green fluorescent protein; TBS, Tris-buffered saline; PI, [^{3}H]phosphoinositide; ERK, extracellular signal-regulated kinase; TAP, tandem affinity purification; MS, mass spectrometry; BP, blood pressure; SBP, systolic BP; WT, wild type; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; mASMC, mouse aortic smooth muscle cell; nNOS, neuronal nitrogen oxide synthase.

In this study, we use biochemical, pharmacological, physiological, and molecular techniques to demonstrate that α-syntrophin is an essential GIP for α_{1D}-AR-binding site formation and coupling to signaling pathways *in vitro* and *in vivo*. In addition, proteomic analysis reveals syntrophins scaffold α_{1D}-ARs to the dystrophin-utrophin-cytoskeleton network, which allows for precise control over α_{1D}-AR functional expression *in vivo*.

EXPERIMENTAL PROCEDURES

Constructs—Mouse α-syntrophin in pBlu2SKP was kindly provided by Dr. Stan Froehner (University of Washington, Seattle, WA). Human α_{1A}- and α_{1B}-AR in pREP4 were made as described (28). Human α_{1D}-AR was cloned into pcDNA3.1/H^+ from pEGFP using KpnI/NheI, followed by QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). To generate α_{1D}-6G and α_{1D}-12G, FLAG-α_{1D}-AR was amplified with PCR to add 5′ KpnI and 3′ EcoRI sites for subcloning into pcDNA3.1 and to remove the α_{1D}-AR stop codon. 6G- or 12G-α-syntrophin was then amplified using PCR to add 5′ EcoRV and 3′ XbaI for subcloning into pcDNA3.1 containing α_{1D}-AR. To generate the α_{1D}-12G PDZ-binding motif mutant, QuikChange site-directed mutagenesis was used to mutate ^{568}RETDI^{572} in α_{1D}-AR to ^{568}AAAAA^{572}. α_{1D}-6G truncations were generated by stop codons at amino acid positions 447 (SU N-stop), 403 (PH2 C-stop), and 286 (PH2 N-stop) using QuikChange site-directed mutagenesis. pIRESpuro-GLUE was kindly provided by Dr. Randy Moon (University of Washington, Seattle, WA). Human α_{1A}-AR, α_{1D}-AR, and α-syntrophin were amplified by PCR to add 5′ EcoRI and 3′ BglII for subcloning into pIRESpuro-GLUE.

Cell Culture and Transfection—HEK293 cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37 °C in 5% CO_2. The constructs were transfected using Lipofectamine 2000 (Invitrogen) when cells were ~70% confluent. Polyclonal stable cell lines were generated with 400–800 mg/ml geneticin or 100–200 μg/ml hygromycin (Invitrogen). Stable cells were maintained with 100 μg/ml geneticin and/or 25 μg/ml hygromycin.

Antibodies—Utrophin antibody 1862 and syntrophin antibodies 1351 (p-syn), syn17 (α), syn248 (β_1), and syn28 (β_2) have been previously described (29). Anti-hemagglutinin (Cell Signaling Tech., Danvers, MA) and anti-β-actin (AbCam, Cambridge, MA) were used according to the manufacturers' instructions. Primary antibodies were detected with IRDye 800CW goat anti-rabbit or IRDye 680 goat anti-mouse (LiCor Biotechnology, Lincoln, NE) and imaged with an Odyssey Scanner (LiCor Biotechnology).

Immunocytochemistry—The cells were co-transfected with α_{1D}-AR-GFP and DsRed-tagged proteins specifically recognizing endoplasmic reticulum, mitochondria, or peroxisome (Invitrogen). The cells were fixed with 4% paraformaldehyde, mounted with Vectashield (containing 4′,6′-diamino-2-phenylindole stain), and sealed with nail polish. The cells were imaged on a Zeiss 510 META confocal microscopy in the W. M. Keck Center (University of Washington, Seattle, WA). HEK293 cells were transfected with Myc-α_{1D}-AR alone or with α-syntrophin-V5, fixed with 4% paraformaldehyde, washed three times with 0.1% Triton X-100 in TBS, blocked/solubilized (TBS, 0.1% Triton X-100, 2% bovine serum albumin, 5% horse serum), and incubated overnight with anti-Myc (1:50, mouse) (Santa Cruz, Biotechnology, Santa Cruz, CA). The cells were washed three times with TBS with 0.05% Tween 20, incubated with 488 goat anti-mouse (Invitrogen), washed three times with TBS with 0.05% Tween 20, washed twice with TBS, and then mounted with Vectashield and sealed with nail polish. The cells were imaged on a Leica SL confocal microscopy in the W. M. Keck Center.

Radioligand Binding—Radioligand binding was assayed as previously described (20). Whole cell membranes were harvested, and total α_1-AR-binding site density was measured with [^{3}H]prazosin (PerkinElmer Life Sciences). Nonspecific binding was determined in the presence of 10 μM phentolamine. Protein was collected with a cell harvester (Brandel, Gaithersburg, MD), and samples were counted with a Packard Tri-Carb 2200 CA liquid scintillation analyzer (Packard Instrument Co. Inc., Rockville, MD).

[^{3}H]Phosphoinositol (PI) Hydrolysis—PI hydrolysis was measured as previously described (20). The cells were incubated with 1 μCi of [^{3}H]*myo*-inositol (American Radiolabeled Chemicals Inc., St. Louis, MO) for 24–48 h, washed, and incubated with phenylephrine (PE) in KHB (129 mM NaCl, 5.5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 20 mM $NaHCO_3$, 11 mM glucose, 0.029 mM Na_2EDTA, 10 mM LiCl) for 1 h. Ion exchange columns were used to separate hydrolyzed [^{3}H]phosphoinositides. The samples were normalized by counting incorporated [^{3}H]*myo*-inositol. The samples were counted with a Packard Tri-Carb 2200 CA liquid scintillation analyzer (Packard Instrument Co. Inc.), and the data were analyzed using Prism Software (GraphPad Software, San Diego, CA).

ERK1/2 Activation—ERK1/2 activation was assayed in a 96-well plate as previously described (30). The cells were stimulated with epidermal growth factor (1 nM, 5 min) or PE (10 min), fixed in 4% paraformaldehyde, washed with blocking buffer (TBS, 0.1% Triton X-100, 2% bovine serum albumin, 5% horse serum) for 1.5 h, and incubated with anti-ERK and anti-pERK in TBS (w/2% bovine serum albumin) overnight at 4 °C. The cells were washed with 0.1% Tween 20 in TBS and incubated with IRDye secondary antibodies. The cells were imaged with LiCor Odyssey system.

TAP Purification—For TAP-α-syntrophin, TAP purification was conducted as previously described (31). Briefly, the cells were lysed overnight and solubilized protein was incubated with streptavidin-Sepharose (GE Healthcare) for 2 h, washed, and eluted with 50 mM D-biotin. Eluate was incubated for 1.5 h with calmodulin-Sepharose (GE Healthcare). Bound Sepharose was washed, and final protein was eluted with 50 mM ammonium bicarb (pH 8.0) + 25 mM EGTA. For TAP-α_{1D}-AR, TAP purification was conducted as previously described (32). The cells were lysed overnight in buffer containing 1% digitonin, and solubilized protein was incubated with streptavidin-Sepharose, washed, and eluted with 50 mM D-biotin. Eluate was incubated with calmodulin-Sepharose, washed, and eluted with 50 mM ammonium bicarb (pH 8.0) +

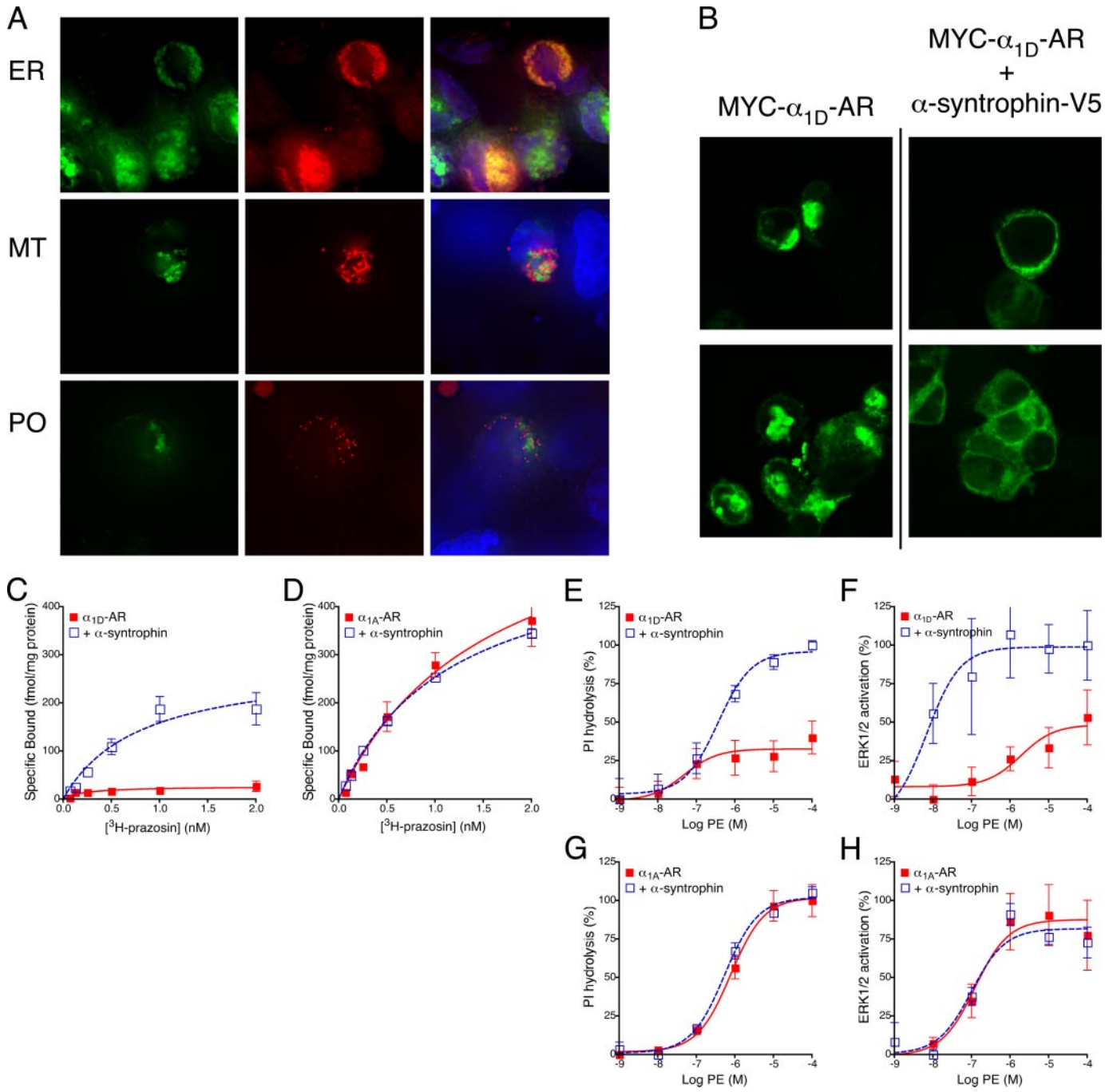

FIGURE 1. **α_{1D}-ARs require syntrophin for functional expression *in vitro*.** *A*, α_{1D}-AR-GFP localization (*green*) was examined in HEK293 cells co-stained with markers (expressing dsRed) for mitochondria (*MT*), peroxisome (*PO*), or endoplasmic reticulum (*ER*). *B*, Myc-α_{1D}-AR (*green*) localization with and without α-syntrophin-V5. *C* and *D*, α-syntrophin specifically increases α_{1D}-AR-binding site density. [^{3}H]prazosin radioligand binding was measured in WT or α-syntrophin-overexpressing HEK293 cells co-transfected with α_{1D}-AR (*C*) or α_{1A}-AR (*D*). *E–H*, α-syntrophin increases agonist stimulated α_{1D}-AR coupling to PI hydrolysis (*E*) and ERK1/2 activation (*F*) but has no effect on α_{1A}-AR functional responses (*G* and *H*). The results are the means ± S.E. of two to four experiments performed in triplicate.

25 mM EDTA. Final eluate was analyzed via mass spectrometry (linear ion trap Fourier transform or LTQ Orbitrap).

Mouse α_{1D}-AR Antibody Production—Glutathione *S*-transferase fusion peptides were constructed with either amino acids 1–23 or 35–59 of the N terminus of α_{1D}-AR for injection into rabbits (R & R Rabbitry, Stanwood, WA). Sera was used at a concentration of 1:100 for Western blotting after confirming specificity.

Protein Isolation—Harvested aortas or cells grown in culture were lysed with low salt lysis buffer (10 mM NaH_2PO_4, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing protease inhibitors and homogenized overnight at 4 °C. Insoluble protein was cleared by centrifugation, and syntrophin was immunoprecipitated from soluble extract with pan-syntrophin ab. Protein complexes were precipitated using protein G-Sepharose. Eluted samples were run on 10% bis-tris gels and probed as indicated.

TABLE 1

Syntrophins specifically increase α_{1D}-AR functional expression *in vitro*

α_{1A}- and α_{1D}-AR-binding site density, PI hydrolysis, and ERK1/2 activation were measured in WT and syntrophin-overexpressing HEK293 cells. Maximal responses for α_{1A}-AR expressing cells are normalized to α_{1A}-AR in WT HEK293 cells, and maximal responses for α_{1D}-AR are normalized to α_{1D}-AR in α-syntrophin-overexpressing HEK293 cells. The data are the means ± S.E. of two to four experiments performed in triplicate.

		B_{max}	S.E.	K_D	S.E.	PI hydrolysis				ERK1/2 activation			
						Log EC_{50}	S.E.	Max	S.E.	Log EC_{50}	S.E.	Max	S.E.
		fmol/mg protein		*nM*		*M*		%		*M*		%	
α_{1A}-AR	HEK293	674.9	148.1	1.56	0.615	−6.1	0.14	102.0	5.25	−6.9	0.35	87.6	9.65
	+ α-syn	541.7	28.1	1.14	0.118	−6.3	0.07	102.0	2.67	−7.0	0.22	81.7	5.54
α_{1D}-AR	HEK293	26.6	7.5	0.22	0.206	−7.3	0.66	32.6	5.84	−5.7	0.66	48.4	11.03
	+ α-syn	285.2	51.7	0.80	0.326	−6.4	0.19	95.9	5.71	−8.2	0.56	98.8	12.85

Blood Pressure—All of the animal studies conducted were approved by IACUC. For heart rate and blood pressure measurements, conscious mice were placed into a CODA 6+ system (Kent Scientific Co., Torrington, CT) with an attached tail cuff. For vasopressor challenge experiments, 12–16-week-old male mice (20–28 mg) were anesthetized with 50 mg/kg sodium pentobarbital intraperitoneally and placed into CODA 6+ system for continuous measurement of BP. The mice were then given either saline, 2.5 μg of prazosin, or 2.5 μg of BMY 7378 intraperitoneally (total volume of injections, 200 μl) followed by 3.12 μg of PE 10 min later. Base line was determined as the average blood pressure (BP) measurements prior to PE injection. The data were analyzed using Prism Software (GraphPad Software).

Aortic Smooth Muscle Cell Isolation—mASMCs were isolated as described (33). Briefly, dissected aortas were incubated in HBSS+ buffer (16.4 mM $NaHCO_3$, 1.7 mM $CaCl_2$, 100 μg/ml streptomycin, 100 units/ml penicillin, 0.73 units/ml elastase, 7000 units/ml soybean trypsin inhibitor, and 265 units/ml collagenase in Hanks HBSS 1× without calcium/magnesium) for 10–15 min. Adventitial and medial layers were separated, and the medial layer was minced and digested in HBSS+ buffer for 60–70 min. The samples were then retriturated and centrifuged gently at 3000 rpm. The supernatant was discarded, and the cells were grown in M199 medium containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Ca^{2+} Mobilization—mASMCs were washed with imaging buffer (75 mM NaCl, 1.5 mM KCl, 1.32 mM $CaCl_2$, 2 mM $MgSO_4$, 2.5 mM HEPES, 10 mM glucose, pH 7.35) and then incubated with 5 μM Fluo-4 AM dye (Invitrogen) diluted in imaging buffer for 30 min at 37 °C. The cells were stimulated with either 100 μM PE or 100 μM UTP and imaged with an inverted Nikon in the W. M. Keck Imaging Center. The data were quantified using Metamorph (Molecular Devices, Sunnyvale, CA) and Prism (GraphPad Software).

RESULTS

α-Syntrophin Increases α_{1D}-AR Functional Expression—Although much information exists on the α_{1A}- and α_{1B}-AR subtypes, the α_{1D}-AR has been poorly studied because of endoplasmic reticulum retention in heterologous expression systems (Fig. 1*A*). Recently, we identified α-syntrophin as a potential α_{1D}-AR GIP through a yeast two-hybrid screen (20). To address the potential importance of α-syntrophin as an α_{1D}-AR GIP, we examined the effects of α-syntrophin overexpression on α_{1D}-AR subcellular localization (Fig. 1*B*). As expected, Myc-α_{1D}-AR expressed in HEK293 cells is retained intracellularly. However, co-expression of Myc-α_{1D}-AR with α-syntrophin facilitates α_{1D}-AR expression at the plasma membrane (Fig. 1*B*). To determine how α-syntrophin effects α_{1D}-AR function, we examined α_{1D}-AR-binding site density and signaling in HEK293 cells. α_{1D}-AR transfection into α-syntrophin cells resulted in a ~10-fold increase in binding site density measured with the α_1-AR-selective antagonist [^{3}H]prazosin (Fig. 1*C* and Table 1). α-Syntrophin had no effect on α_{1A}-AR (Fig. 1*D* and Table 1) or α_{1B}-AR (data not shown) binding site density. Additionally, α-syntrophin overexpression specifically enhanced PE potencies (EC_{50}) and maximal responses for stimulating PI production and ERK1/2 phosphorylation (Fig. 1, *E–H*, and Table 1). Taken together, these findings demonstrate that α-syntrophin is essential for α_{1D}-AR functional expression *in vitro*.

The SU Domain of α-Syntrophin Is Essential for α_{1D}-AR Functional Expression—α-Syntrophin contains a PDZ domain that recognizes the consensus PDZ-binding motif (RETDI) located within the α_{1D}-AR distal C terminus (26). Interestingly, α-syntrophin contains a PDZ domain that is flanked by a split PH domain and contains a second PH2 domain and a SU domain important for protein scaffolding (21, 24, 34). To examine the importance of each domain for α_{1D}-AR signalosome function, α-syntrophin was tethered to the α_{1D}-AR C terminus with a six- (6G) or twelve-glycine residue linker (12G) to permit flexibility (Fig. 2*A*). This method forces α-syntrophin to interact with α_{1D}-AR, prevents other PDZ-containing proteins from interacting with the α_{1D}-AR PDZ-binding motif, and ensures a 1:1 expression ratio between α_{1D}-AR and α-syntrophin (35). Tethering syntrophin to α_{1D}-AR with either a 6G (α_{1D}-6G) or 12G (α_{1D}-12G) linker caused a robust increase in α_{1D}-AR-binding sites and functional responses (Fig. 2, *B–D*). Alanine mutagenesis of the PDZ-binding motif in α_{1D}-AR (^{568}RETDI^{572} → ^{568}AAAAA^{572}) eliminated α_{1D}-12G stimulated [^{3}H]PI hydrolysis (Fig. 3*B*), demonstrating the critical importance of this domain for forming the α_{1D}-AR/α-syntrophin signalosome. Next, truncation mutants were used to assess the importance of the PH2 and SU domains of syntrophin for α_{1D}-AR signalosome function (Fig. 3*A*). The absence of the SU domain markedly decreased α_{1D}-6G functional expression, and additional truncations did not enhance this effect (Fig. 3*B* and Table 2), suggesting that the SU domain is of critical importance for α_{1D}-AR signalosome assembly.

The α_{1D}-AR Signalosome Includes Multiple Members of the Dystrophin Complex—α-Syntrophin acts as a scaffold for many proteins (*i.e.* AQP4, nNOS, and plasma membrane calcium ATPase1/4 (21, 24, 34)), facilitating proper spatial and temporal organization of multi-protein complexes within cells. To address the possibility that α-syntrophin scaffolds additional GIPs to the α_{1D}-AR signalosome, we performed TAP/MS analysis using α-syntrophin as bait. TAP/MS of α-syntrophin identified proteins involved in signal transduction, endoplasmic reticulum quality control, protein degradation, and cytoskeletal organization (Table 3). As expected, multiple members of the dystrophin complex including utrophin, dystrobrevin, and dystrophin were identified as α-syntrophin interacting partners. Additionally, we identified proteins previously suggested to be important for α_{1D}-AR function *in vivo*, including PLCβ3 and Na^+/PO_4^- cotransporter (36). We then performed the reverse experiment using the α_{1D}-AR as bait. Multiple members of the dystrophin complex were identified as α_{1D}-AR-interacting proteins including utrophin, dystrobrevin, and multiple syntrophin isoforms (Table 3). Interestingly, β_1- and β_2-syntrophin were identified, but not α-syntrophin, which can be explained by the endogenous expression of β_1- and β_2-syntrophin and not α-syntrophin in HEK293 cells (Fig. 4*A*). Next, we tested the validity of our TAP/MS results by immunoprecipitating TAP-α_{1D}-AR and prob-

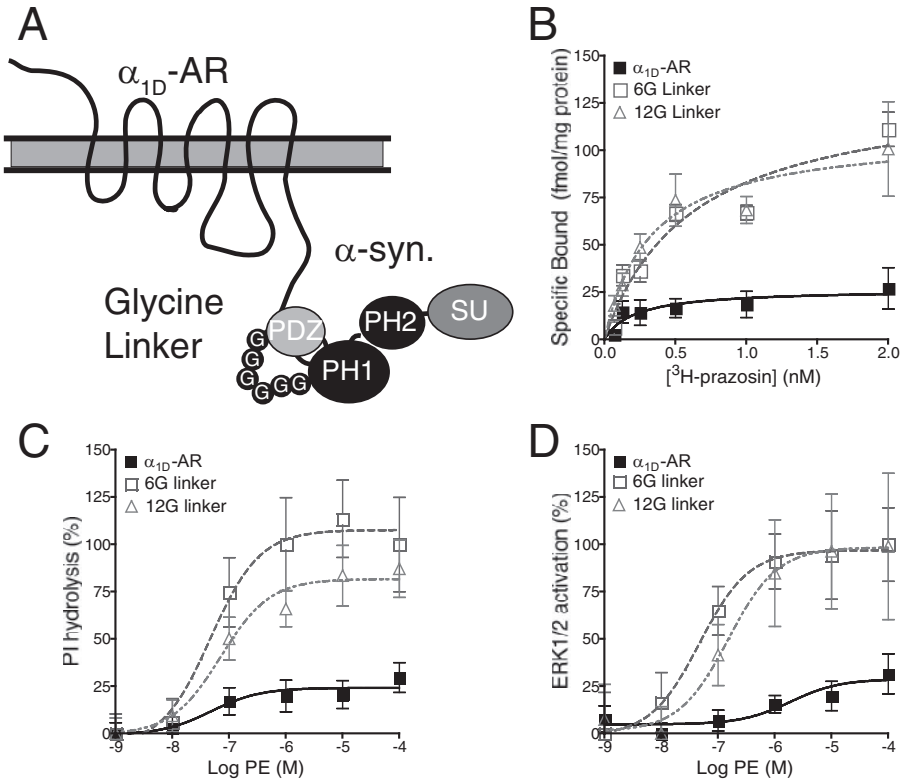

FIGURE 2. **Characterization of the α_{1D}-AR/α-syntrophin linker constructs.** *A*, schematic of the α_{1D}-AR-6G-α-syntrophin fusion construct (α_{1D}-6G). *B–D*, the ability of the α_{1D}-6G and 12G fusion proteins to form functional binding sites was determined by [^{3}H]prazosin saturation radioligand binding (*B*) and the ability to couple to agonist-stimulated PI hydrolysis (*C*) and ERK1/2 activation (*D*) in HEK293 cells. The data were normalized to maximal α_{1D}-6G linker responses and are the means ± S.E. of two or three experiments performed in triplicate.

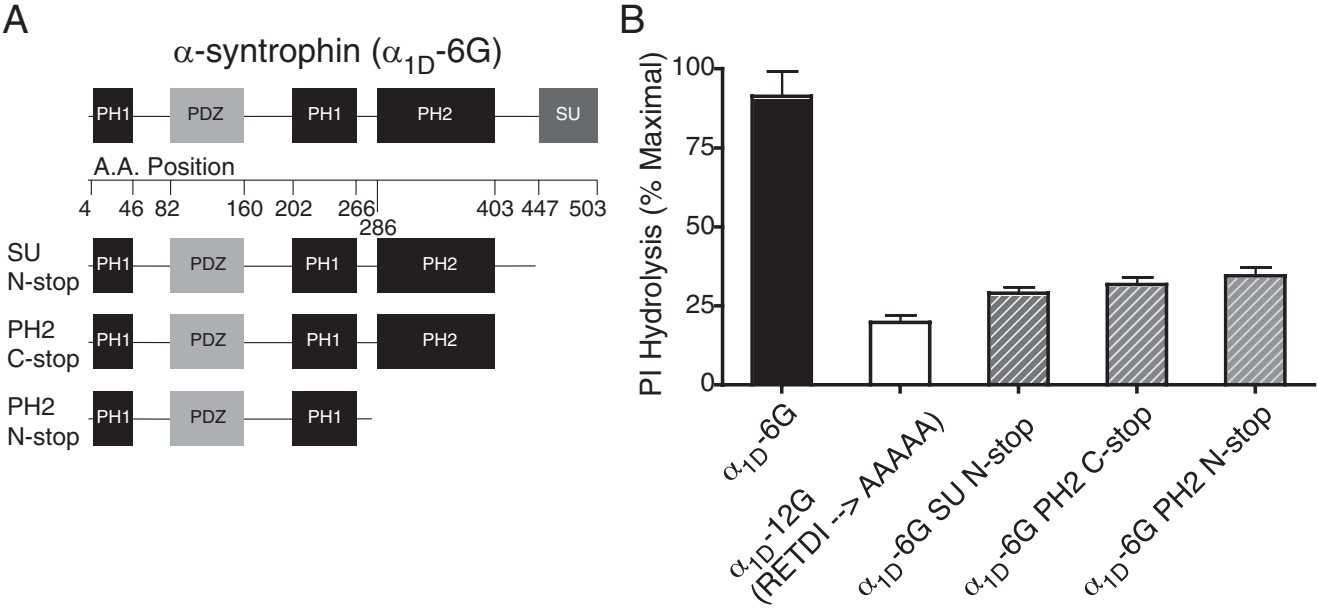

FIGURE 3. **The SU domain of α-syntrophin is required for α_{1D}-AR function.** *A*, schematic of α_{1D}-6G deletion constructs. Stop codons were introduced before the SU domain (SU N-stop), after the PH2 domain (PH2 C-stop) and before the PH2 domain (PH2 N-stop). *B*, quantification of agonist-stimulated PI hydrolysis by α_{1D}-6G, the ΔPDZ-binding motif in α_{1D}-12G (RETDI → AAAAA), and α_{1D}-6G deletion constructs. The responses are normalized to maximal responses stimulated by α_{1D}-6G and are the means ± S.E. of three experiments performed in triplicate.

TABLE 2

Deletion of SU-PH2 domain of syntrophin decreases α_{1D}-AR PI hydrolysis

HEK293 cells were transiently transfected with either the α_{1D}-6G, Δ PDZ-binding motif in α_{1D}-12G or α_{1D}-6G truncations. PE-mediated PI hydrolysis was measured, and log EC_{50} and maximal responses are shown. The data are normalized to α_{1D}-6G and represent three experiments performed in triplicate.

Construct	Log EC_{50}	S.E.	Max response	S.E.
	M		%	
6G linker	−7.04	0.32	91.4	7.9
ΔPDZ binding motif	−6.60	0.55	19.8	2.3
SU N-stop	−6.83	0.23	29.8	2.2
PH2 C-stop	−7.04	0.25	31.8	2.3
PH2 N-stop	−7.70	0.30	34.5	2.7

TABLE 3

Tandem affinity purification results for α-syntrophin and α_{1D}-AR

TAP-α-syntrophin and TAP-α_{1D}-AR purified from HEK293 cells were analyzed by mass spectrometry. For each protein identified, the number of separate peptide sequences identified through MS (Hits) and the overall peptide coverage in the amino acid sequence are shown (Coverage). The masses of identified proteins are also shown (Mass).

	Protein name	Hits	Coverage	Mass
			%	*kDa*
TAP-α-syntrophin purification	α-Syntrophin	10	21	54
	Dystrobrevin α	3	6	84
	Dystrophin	4	1	423
	Gustducin α-3	1	8	38
	Na^+/PO_4^+ co-transporter type IIc	1	6	63
	Nebulin	5	1	773
	PLCβ−3	1	1	139
	RGS11	1	6	22
	Ryanodine receptor 2	2	1	546
	Signal transducing adaptor protein 1	1	3	34
	Utrophin	12	5	394
TAP-α_{1D}-AR purification	α_{1D}-AR	3	4	60
	Actin (aortic smooth muscle type)	1	4	42
	Autocrine motility factor receptor	5	8	73
	Cytoskeleton-associated protein 4	16	35	66
	Dystrobrevin α	3	6	84
	ERLIN 1	4	16	39
	ERLIN 2	19	48	39
	GOPC	12	28	51
	β_1-Syntrophin	12	26	58
	β_2-Syntrophin	9	19	58
	Utrophin	3	2	394

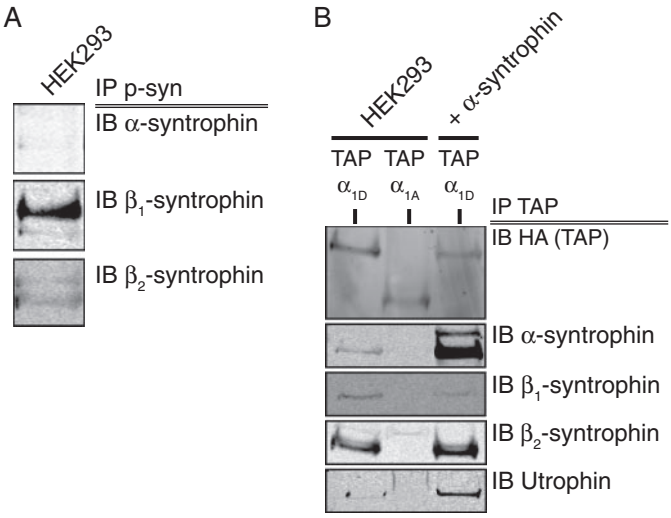

FIGURE 4. **Characterization of the α_{1D}-AR/α-syntrophin signalosome.** *A*, HEK293 cells express β_1- and β_2-syntrophin isoforms. HEK293 cell lysate was immunoprecipitated with a pan-syntrophin antibody (*p-syn*) and was probed with anti-syntrophin isoform specific antibodies. *B*, syntrophins recruit utrophin into the α_{1D}-AR signalosome. HEK293 cell lysates expressing TAP-α_{1D}-AR were immunoprecipitated with streptavidin and blotted for hemagglutinin (located within the TAP-tag), syntrophin isoforms and utrophin. TAP-α_{1A}-AR is unable to associate with syntrophin/utrophin (*middle lane*). TAP-α_{1D}-ARs associate with syntrophins/utrophins in WT cells (*left lane*), and the interaction is increased in α-syntrophin-overexpressing cells (*right lane*). *IB*, immunoblot.

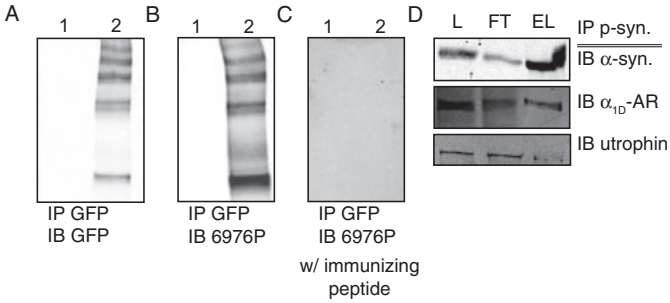

FIGURE 5. **The α_{1D}-AR/α-syntrophin signalosome forms *in vivo*.** *A*, mouse α_{1D}-AR-GFP was immunoprecipitated (*IP*)/immunoblotted (*IB*) with antibodies directed against GFP. α_{1D}-AR-GFP was precipitated from HEK293 cells expressing α_{1D}-AR-GFP (*lane 2*) but not in untransfected HEK293 cells (*lane 1*). *B* and *C*, α_{1D}-AR-GFP was immunoprecipitated from HEK293 cells (*lane 2*) and blotted for with our in-house rabbit anti-mouse-α_{1D}-AR antibody (6976P). 6976P recognizes α_{1D}-AR-GFP in transfected HEK293 cells (*B*, *lane 2*) but not in untransfected HEK293 cells (*B*, *lane 1*). The ability of 6976P to detect α_{1D}-AR-GFP was blocked by pretreating the antibody with immunizing peptide (*C*), confirming its specificity. *D*, aortic smooth muscle lysates were immunoprecipitated with a pan-syntrophin antibody (*p-syn*) and probed for α-syntrophin (*top row*), α_{1D}-AR (*middle row*) or utrophin (*bottom row*). *L*, lysate load on beads; *FT*, flow through collected; *EL*, eluate collected from beads.

ing for dystrophin complex members. TAP-α_{1D}-AR interacted with utrophin and β_1/β_2-syntrophin (Fig. 4*B*), and immunoprecipitating TAP-α_{1D}-AR from α-syntrophin-overexpressing cells increased the recruitment of utrophin to the α_{1D}-AR signalosome (Fig. 4*B*).

The α_{1D}-AR/α-Syntrophin Signalosome Forms in Vascular Smooth Muscle—α_{1D}-ARs are key regulators of cardiovascular system function, where they regulate total peripheral resistance by contracting vascular smooth muscle (1). Interestingly, the majority of blood vessels express a mixture of α_{1A}-, α_{1B}-, and α_{1D}-AR subtypes, whereas the mouse aorta is the only blood vessel that displays a pure population of functional α_{1D}-AR (37, 38). To determine whether the α_{1D}-AR/syntrophin/utrophin signalosome forms in the mouse aorta, we created an antibody directed against the N-terminal domain of the mouse α_{1D}-AR. To test the specificity of our α_{1D}-AR antibody, C-terminal GFP-tagged mouse α_{1D}-AR was transfected into HEK293 cells and subjected to immunoprecipitation/immunoblotting. Antibodies directed against GFP selectively recognized both monomers and multi-order oligomers (Fig. 5*A*) in agreement with previous studies (39, 40). Antibody 6976P (Fig. 5*B*) or 6895P (data not shown) specifically recognized GFP-α_{1D}-AR following GFP immunoprecipitation, which was blocked by pretreating with immunizing peptide (Fig. 5*C*). Next, mouse aortas were prepared into cell lysate, immunoprecipitated for syntrophin (p-syn), and then probed for α_{1D}-AR and utrophin. Fig. 5*D* demonstrates that α_{1D}-AR co-immunoprecipitated with both α-syntrophin and utrophin in aortic cell lysate, demonstrating the *in vivo* relevance of the α_{1D}-AR/syntrophin/utrophin signalosome.

The α_{1D}-AR/Syntrophin Signalosome Regulates Systolic Blood Pressure—Previous studies have shown an approximate 10% decrease in resting BP in α_{1D}-AR KO mice (4, 37, 38). If syntrophin is essential for α_{1D}-AR function, we expect that

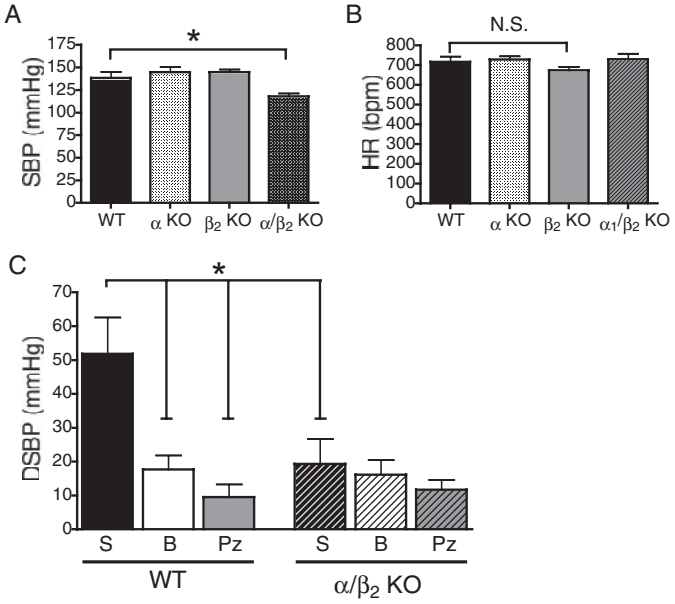

FIGURE 6. **α- and β_2-Syntrophin are both required for α_{1D}-AR mediated blood pressure responses.** *A*, resting blood pressure is abrogated in α/β_2-syntrophin KO mice. *B*, systolic blood pressure and heart rates (*HR*) were measured by tail cuff in conscious mice. * indicates significant difference as determined by unpaired *t* test ($p < 0.05$). *C*, PE-stimulated increases in SBP are eliminated in α/β_2-syntrophin KO mice. The mice were pretreated with saline (*S*), BMY 7378 (*B*), or prazosin (*Pz*) prior to PE injection. All of the recordings are the means ± S.E. of six to nine mice/group.

syntrophin KO mice will display similar decreases in resting BP. Interestingly, resting systolic BP (SBP) and heart rates in α-syntrophin KO and β_2-syntrophin KO mice were equivalent to those observed in WT mice (Fig. 6, *A* and *B*). However, α/β_2-syntrophin double KO mice displayed a markedly hypotensive phenotype during rest (Fig. 6*A*) similar to that reported in α_{1D}-AR KO mice (4). These findings show that syntrophin isoforms can compensate for each other, in agreement with previous studies (34).

We hypothesized that the decrease in SBP observed in α/β_2-syntrophin KO mice is the result of compromised α_{1D}-AR function. To test this, the mice were injected with PE, and resulting increases in SBP were recorded. As expected, WT mice displayed robust increases in SBP in response to PE challenge, which was inhibited by pretreatment with either prazosin or BMY 7378. Interestingly, the ability of PE to increase SBP in α/β_2-syntrophin double KO mice was markedly diminished (Fig. 6*B*) and was similar to that observed in WT mice when administered PE + BMY 7378. The findings that deletion of α- and β_2-syntrophin compromises α_{1D}-AR-stimulated vasoconstriction demonstrate the importance of these syntrophins for proper α_{1D}-AR function in vascular smooth muscle. This effect was further demonstrated upon examination of α_{1D}-AR function in primary cultures of mouse aortic smooth muscle cells. Mouse aorta express both α- and β_2-syntrophin (Fig. 7*A*), and α_{1D}-AR-stimulated $[Ca^{2+}]_i$ is eliminated in cells isolated from α/β_2-syntrophin KO mice (Fig. 7, *B* and *C*). Responses for the $G\alpha_{q/11}$-coupled receptor P2Y remained intact (Fig. 7*C*, *inset*), indicating that the loss in α_{1D}-AR functional responses is a direct result of syntrophin deletion. Taken together, these data demonstrate that the α_{1D}-AR/syntrophin signalosome forms *in vivo* to mediate contraction of vascular smooth muscle and regulate BP and that multiple syntrophin isoforms are capable of promoting α_{1D}-AR signalosome assembly.

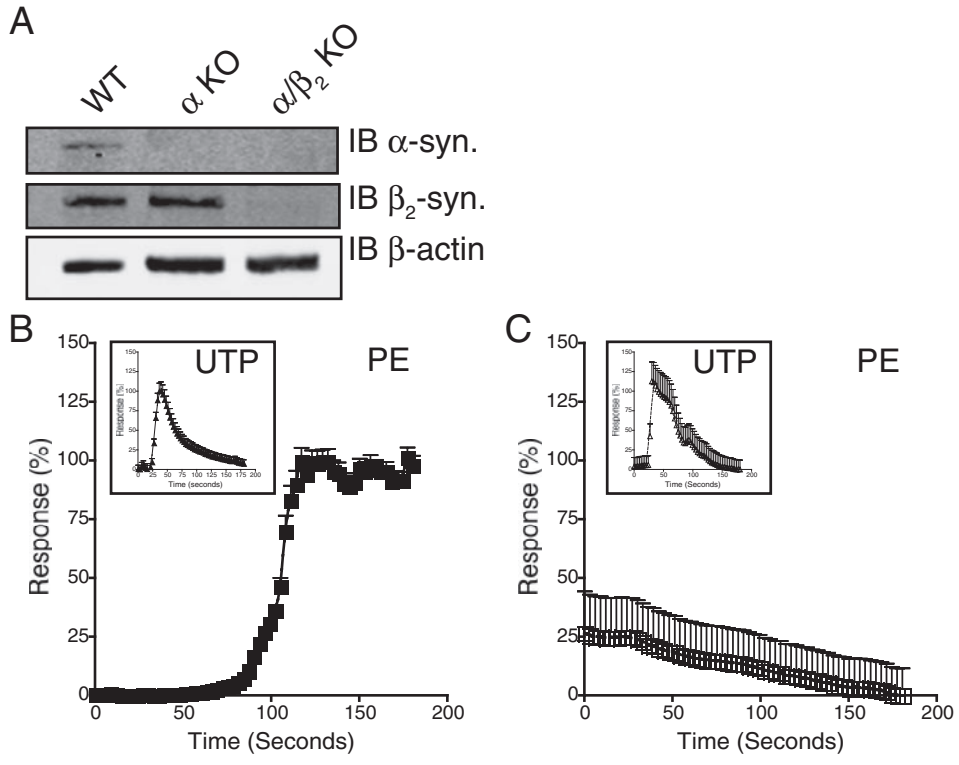

FIGURE 7. **α_{1D}-AR mediated Ca^{2+} mobilization requires syntrophins.** *A*, aortic smooth muscle cells express the α- and β_2-syntrophin isoforms. Cell lysates from WT, α-syntrophin, and α/β_2-syntrophin KO mice were probed with anti-syntrophin isoform-specific antibodies. *B* and *C*, α_{1D}-AR functional responses are eliminated in ASMCs from α/β_2-syntrophin KO mice. Freshly dissociated ASMCs were isolated from WT (*B*) and α/β_2-syntrophin (*C*) mice, and Ca^{2+} mobilization was measured in response to 100 μM PE and 100 μM UTP (*inset*). The data were normalized to maximal WT responses. *IB*, immunoblot.

DISCUSSION

The results of this study contribute to the continuing evolution of GPCR identity. Originally perceived to be simple seven-transmembrane spanning polypeptides coupled to a single G-protein, we now know that GPCRs are dynamic multi-protein complexes that undergo constant transition in components and structure to regulate expression, trafficking, and functional coupling. The processes that occur after GPCR activation, specifically desensitization, internalization, recycling, and/or degradation, have been thoroughly characterized (12). For example, the association of regula-

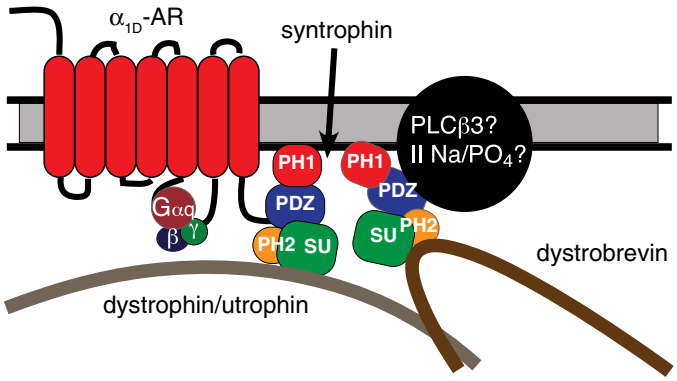

FIGURE 8. **Proposed model of the α_{1D}-AR/syntrophin signalosome.** Syntrophins anchor α_{1D}-AR at the plasma membrane through interactions with dystrophin-utrophin and dystrobrevin. The dystrophin-utrophin complex can bind up to four syntrophins, allowing other syntrophins in the complex to scaffold additional regulators of signal transduction in close proximity to α_{1D}-AR (*i.e.* PMCA, nNOS, TRPC, PLCβ3, and/or RGS11).

tory proteins (*i.e.* arrestins, dynamin, G-protein coupled receptor kinases) in a temporally and spatially specific manner with GPCRs permits specificity of function and precise regulation of GPCR signaling (41). Here, we highlight that the formation of signalosomes are important for events before agonist stimulation for certain GPCRs. Using a combination of molecular, pharmacological, proteomic, and physiological experiments, we clearly demonstrate that syntrophins anchor α_{1D}-ARs at the plasma membrane in a PDZ domain-dependent manner as a multi-protein complex. Disruption of this essential protein-protein interaction prevents proper α_{1D}-AR assembly, functional coupling, and regulation of cardiovascular system function.

Although the α_{1D}-AR is a key regulator of cardiovascular function (3, 4), few studies have investigated the molecular characteristics of this important receptor. This is due to the difficulties in obtaining sufficient expression and function when expressed *in vitro*, because unlike the closely related α_{1A}- and α_{1B}-AR subtypes, the α_{1D}-AR is retained intracellularly. Previously, N-terminal truncation or heterodimerization with other GPCRs have been demonstrated to facilitate α_{1D}-AR functional expression (11, 28, 42, 43). These studies add an additional layer of complexity to the α_{1D}-AR signalosome. Unfortunately, we do not yet have the necessary tools to confirm that these mechanisms are actually occurring *in vivo* and how this information can be incorporated into our growing knowledge of α_{1D}-AR function.

Based on the results of our TAP/MS screen, we propose that syntrophin acts as an adaptor that links the α_{1D}-AR to the dystrophin-utrophin-cytoskeleton network (Fig. 8). In this model syntrophins anchor α_{1D}-AR at the plasma membrane through interactions with dystrophin-utrophin and dystrobrevin. Indeed, deletion of the SU domain, which anchors syntrophin to dystrophin, utrophin, and dystrobrevin (44), results in loss of α_{1D}-AR function. The dystrophin-utrophin complex can bind up to four syntrophins, allowing other syntrophins in the complex to scaffold additional regulators of signal transduction in close proximity to α_{1D}-AR (*i.e.* plasma membrane calcium ATPase, nNOS, and/or TRPC) (21, 24, 34). Furthermore, our TAP/MS analysis of α-syntrophin revealed numerous signal transduction components (*i.e.* PLCβ3, ryanodine receptor, gustducin, and Na^+/PO_4^- co-transporter) or regulators of function (*i.e.* RGS11), suggesting that α-syntrophin acts as a scaffold for diverse cellular proteins. The Na^+/PO_4^- co-transporter has been previously linked to α_1-AR function in the heart (36, 45, 46), and it may prove interesting to examine whether α-syntrophin facilitates the co-localization of the Na^+/PO_4^- co-transporter to α_{1D}-AR. In essence, the α_{1D}-AR/dystrophin signalosome forms a signaling microdomain to enable efficient functional coupling by maintaining all of the necessary components within close spatial proximity. Furthermore, this model explains why overexpression of syntrophins are required to obtain detectable increases in α_{1D}-AR-binding site density and functional coupling, because the level of endogenous syntrophins in heterologous systems is the rate-limiting factor.

The identification of dystrophins as essential GIPs in the α_{1D}-AR signalosome will permit characterization of this important GPCR *in vitro* and *in vivo*. Previously, other receptors have been shown to require obligate GIPs to permit functional expression, including those that form heterodimers (*i.e.* T1R1/T1R2 taste receptors and $GABA_BR1/R2$ (47, 48)), those that interact with single, transmembrane spanning peptides (*i.e.* RAMPs/CGRP and Nina A/rhodopsin (49, 50)), or those that bind multiple proteins after agonist stimulation (*i.e.* β_2-AR/arrestins (41, 51)). Unfortunately, many GPCRs are still unable to be studied *in vitro*, because the GIPs necessary for their proper functional expression remain to be identified. Thus, a continuing effort to identify GIPs necessary for GPCR signalosome function is important for future drug discovery for a number of reasons. Enabling the proper functional expression of GPCRs *in vitro* permits high throughput screening for novel ligands that can be used for the treatment of disease. Also, identifying the interacting domains between GIPs and their cognate GPCRs represents a novel drug target. Discovering molecules that disrupt the interaction between GPCR and GIP could result in a complete loss of receptor function, thus allowing us to develop antagonists with novel mechanisms of action. On the contrary, discovery of molecules that enhance the interaction between a GPCR and GIP may allow us to pharmacologically extend GPCR signalosome lifespan and increase functional coupling. Thus, the characterization of GPCR signalosomes represents an emerging concept in the field of drug discovery.

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