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Syntrophin isoforms play specific functional roles in the α_{1D} -adrenergic receptor/DAPC signalosome

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Abstract

α_{1D} -Adrenergic receptors, key regulators of cardiovascular system function, are organized as a multi-protein complex in the plasma membrane. Using a Type-I PDZ-binding motif in their distal C-terminal domain, α_{1D} -ARs associate with syntrophins and dystrophin-associated protein complex (DAPC) members utrophin, dystrobrevin and α -catulin. Three of the five syntrophin isoforms (α , β_1 and β_2) interact with α_{1D} -ARs and our previous studies suggest multiple isoforms are required for proper α_{1D} -AR function *in vivo*. This study determined the contribution of each specific syntrophin isoform to α_{1D} -AR function. Radioligand binding experiments reveal α -syntrophin enhances α_{1D} -AR binding site density, while phosphoinositol and ERK1/2 signaling assays indicate β_2 -syntrophin augments full and partial agonist efficacy for coupling to downstream signaling mechanisms. The results of this study provide clear evidence that the cytosolic components within the α_{1D} -AR/DAPC signalosome significantly alter the pharmacological properties of α_1 -AR ligands *in vitro*.

Keywords

G-protein coupled receptor; α_1 -adrenergic receptor; Syntrophin; Signalosome; Dystrophin-associated protein complex; Adrenergic; Pharmacology

1. Introduction

α_1 -Adrenergic receptors (AR) are members of the G-protein coupled receptor (GPCR) superfamily that respond to the catecholamines norepinephrine and epinephrine during periods of intense physical activity, stress or injury [1]. Clinically, the α_1 -ARs are important drug targets for cardiovascular disease, benign prostatic hypertrophy and post-traumatic stress disorder [1,2]. Of the three α_1 -AR subtypes, the physiological roles, pharmacological properties and signal transduction mechanisms of the α_{1A} - and α_{1B} -AR subtypes have been the best characterized *in vitro* and *in vivo* [1]. Interestingly, few studies exist examining the

α_{1D} -AR subtype because of the inability to obtain significant expression in cultured cells due to retention within the endoplasmic reticulum [3–5]. Given the importance of this receptor in the cardiovascular system, central nervous system and urinary tract [6,7], understanding the mechanisms controlling α_{1D} -AR functional expression are of critical importance for the development of new therapeutic agents targeting this receptor.

We recently used tandem affinity purification followed by mass spectrometry to reveal α_{1D} -ARs are expressed as a multi-protein complex, or “signalosome” at the plasma membrane [5,8]. α_{1D} -ARs interact with the syntrophin family of scaffolding proteins through a PSD95/DlgA/Zo-1 (PDZ)-domain mediated interaction between the embedded PDZ domain in syntrophins and the PDZ-binding motif in the α_{1D} -AR distal C-terminus [9]. Formation of this complex is obligate for α_{1D} -AR function, as destruction of the PDZ-binding motif in the α_{1D} -AR C-terminus results in loss of α_{1D} -AR drug binding, signal transduction and plasma membrane localization [5,9]. Syntrophins anchor α_{1D} -ARs to the dystrophin-associated protein complex (DAPC), which includes dystrophin, utrophin, dystrobrevins and α -catulin. Through this complex, necessary signaling molecules PLC- β 2 and Rho-GEF are recruited in close proximity to the receptor [8]. This signaling complex is not mimicked by the α_{1A} or α_{1B} -AR subtypes, and has the potential to be used by any of other GPCRs containing PDZ-binding motifs in their C-terminal domains (i.e. 5-HT₂, β -ARs) [10].

Of the five syntrophin isoforms identified (α , β_1 , β_2 , γ_1 , γ_2), three isoforms α -, β_1 - and β_2 -syntrophin interact with α_{1D} -ARs [9]. However, the function of each syntrophin isoform within the α_{1D} -AR/DAPC signalosome remains to be uncovered. Previous studies provide clues; α_{1D} -AR functional responses are unaffected in α - or β_2 -syntrophin knock-out (KO) mice, but are completely ablated in double α/β_2 -syntrophin KO mice [5]. Additionally, syntrophin isoforms demonstrate selectivity in their protein–protein interactions [8]. Taken together, these findings suggest that multiple syntrophin isoforms are clustered within the α_{1D} -AR/DAPC signalosome, and that each performs a specific function within the complex.

Towards addressing this hypothesis, this study examined the possibility that syntrophin isoforms alter the pharmacological and signaling properties of the α_{1D} -AR signalosome. We calculated equilibrium dissociation constants, potencies and intrinsic activities of a number of α_1 -AR selective ligands in cells expressing α_{1D} -ARs with α -, β_1 - or β_2 -syntrophin. Syntrophin isoforms had indistinguishable effects on ligand affinity. However, syntrophin isoforms displayed a consistent rank order on binding site density of $\alpha > \beta_2 > \beta_1$, and on agonist potency with $\beta_2 > \alpha > \beta_1$. Our data support the hypothesis that syntrophin isoforms have unique functional roles within the α_{1D} -AR/DAPC signalosome.

2. Material and methods

2.1. Drugs

[³H]-Prazosin was purchased from Perkin Elmer (Waltham, MS) and [³H]-myo-inositol was purchased from American Radiolabel Chemicals Inc. (St. Louis, MO). 5-MU, A-315456, benoxathian, B MY-7378, cyclazosin, doxazosin, epinephrine, methoxamine, nap hazoline, niguldipine, norepinephrine, phenylephrine, octopamine, synephrine and terazosin were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Constructs

Mouse β_1 -syntrophin and β_2 -syntrophin cDNA in pBlu2SKP was provided by Dr. Stanley Froehner (University of Washington, Seattle, WA). Construction of human α_{1D} -AR and mouse α -syntrophin constructs were previously described in [5].

2.3. Cell culture and transfection

HEK293 cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 mg/mL streptomycin and 100 units/mL penicillin at 37 °C in 5% CO₂. Constructs were transfected using FuGENE HD transfection reagent (Roche Applied Science, Indianapolis, IN) when cells were ~80% confluent. Stable cells were generated by selection with 100 µg/mL geneticin and/ or 25 µg/mL hygromycin.

2.4. Radioligand binding

[³H]-Prazosin saturation and competition assays were performed as previously described [5]. Briefly, whole cell membranes were harvested from HEK293 cells expressing α_{1D} -AR with or without various syntrophin isoforms. Fixed membrane concentrations were incubated with [³H]-prazosin for 30 min at 37 °C, samples were then harvested with a cell-harvester (Brandel, Gaithersburg, MD) and counted with a Packard Tri-Carb 2200 CA liquid scintillation analyzer (Packard Instrument Co. Inc., Rockville, MD). Non-specific binding was determined with 10 µM phentolamine. Competition assays were formed with varying concentrations of antagonist and 1 nM [³H]-prazosin. Data were analyzed with Prism Software (GraphPad Software, San Diego, CA).

2.5. ERK1/2 activation

ERK1/2 activation was assayed in a 96-well plate as previously described [5]. Briefly, HEK293 cells expressing α_{1D} -AR with or without syntrophin isoforms were seeded into a 96-well plate. Cells were serum starved for 24 h, and then stimulated with either phenylephrine (PE) or epidermal growth factor (EGF, positive control) for 10 min. Cells were then fixed with 4% paraformaldehyde, washed with blocking buffer and incubated with anti-ERK and anti-phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. Cells were then washed, incubated with secondary antibody (IRDye 680 and 800 antibodies, LiCor Biotechnology, Lincoln, NE) and imaged on a LiCor Odyssey Scanner. Data were analyzed with Prism GraphPad software.

2.6. [³H]-Phosphoinositol (PI) hydrolysis

PI hydrolysis was measured as previously described [8]. Briefly, cell expressing α_{1D} -AR with or without syntrophin isoforms were seeded into 24-well plates at a density of 1,000,000 cells/well with [³H]-myo-inositol at a concentration of 2 µCi/mL. Cells were incubated overnight at 37 °C, washed with warmed Krebs buffer (w/LiCl) and stimulated with drugs as indicated for 1 h at 37 °C. Buffer was removed, and 800 µl ice-cold methanol was added to each well. Five hundred µl of H₂O was added to each well, and cells were sonicated for 5 s. Samples were transferred to eppendorf tubes and 500 µl chloroform was added. Samples were re-sonicated and then centrifuged at 10,000g for 5 min at 4 °C. One mL of the top, aqueous layer was then added to a pre-washed PI column, washed with 40

mL 5 mM myo-inositol and then eluted with 4 mL Formate/Formic acid mix. Samples were incubated for 1 h at room temperature, then counted on a Packard Tri-Carb 2200 CA liquid scintillation analyzer. Data were analyzed with Prism GraphPad software.

3. Results and discussion

3.1. Effect of syntrophin isoforms on α_{1D} -AR ligand affinity

α -Syntrophin increases α_{1D} -AR localization to the plasma membrane, binding site formation and coupling to signaling responses [5]. However, α -syntrophin KO mice have normal resting blood pressure and intact α_{1D} -AR responses, whereas α -/ β_2 -syntrophin double KO mice are significantly hypotensive and display disrupted α_{1D} -AR function. Since β_1 -/ β_2 -syntrophin both interact with the α_{1D} -AR through a PDZ interaction [9], we wondered if β_1 - and/or β_2 -syntrophin were necessary for α_{1D} -AR functional responses, and if so, how they contributed to signalosome mechanics.

It has been well documented that protein–protein interactions can modify GPCR ligand-binding properties and signaling mechanisms [10]. Thus, we questioned if syntrophin isoforms impart pharmacological differences to the α_{1D} -AR/DAPC signalosome. To test this hypothesis, we measured equilibrium dissociation constants (K_I) for an array of α_1 -AR antagonists using [3 H]-prazosin competition radioligand binding assays on HEK293 cell membranes expressing α_{1D} -AR with α -, β_1 - or β_2 -syntrophin. Representative competition curves are shown for the α_{1D} -AR selective antagonist B MY7378 (Fig. 1A), the α_{1A} -AR selective antagonist 5-methylurapidil (5-MU, Fig. 1B) and the non-selective α_1 -AR antagonist terazosin (Fig. 1C). In all cases, antagonists recognized a single binding site within the range of previously reported K_I values *in vitro* and *in vivo* (Table 1) [11–15]. Thus, these results suggest syntrophin isoforms do not alter the pharmacological properties of the α_{1D} -AR/DAPC signalosome.

3.2. Effect of syntrophin isoforms on α_{1D} -AR binding site density

Next, we compared the ability of each syntrophin isoform to increase α_{1D} -AR binding site density. α -, β_1 - or β_2 -syntrophin were stably co-expressed with α_{1D} -AR in HEK293 cells, lysed into membranes and used for [3 H]-prazosin saturation radioligand analysis. Both β_1 - and β_2 -syntrophin significantly increased α_{1D} -AR binding site density over α_{1D} -AR expressed alone (Table 2, Fig. 2). Surprisingly, syntrophin isoforms displayed significant differences in their Bmax values (α = 285.2 fmol/mg; β_1 = 123.4 fmol/mg; β_2 = 138.4 fmol/mg), with no difference in prazosin affinity (K_D α = 0.8 nM; β_1 = 0.87 nM; β_2 = 0.52 nM). Quantitative RT-PCR and western blotting confirmed syntrophin isoform expression levels were equivalent in each cell line (data not shown). Thus, syntrophin isoforms demonstrate a marked difference in their ability to promote proper folding of α_{1D} -AR binding sites at the plasma membrane, with rank order α -syntrophin > β_2 -syntrophin > β_1 -syntrophin.

3.3. Effect of syntrophin isoforms on α_{1D} -AR agonist efficacy

Given syntrophins differ in their ability to promote α_{1D} -AR binding site density, we suspected these increases would directly correlate with enhanced agonist efficacy. To test our assumption, we generated concentration–response curves for the selective α_1 -AR full

agonist phenylephrine. We measured total inositol phosphate (IP) production using ion exchange chromatography as a functional output, which is downstream of the canonical G α q/11 signaling pathway. Again, we used the same HEK293 cell lines used in our radioligand binding studies, which stably express α_{1D} -ARs with either α , β_1 or β_2 syntrophin. As shown in Fig. 3A, phenylephrine produced sigmoidal increases in IP production in syntrophin overexpressing cells relative to cells expressing α_{1D} -ARs alone. However, phenylephrine maximal responses (or intrinsic activity) did not correlate with the ability of syntrophin isoforms to increase α_{1D} -AR binding site density. Instead, β_2 -syntrophin caused a dramatic increase in phenylephrine maximal responses, while α -syntrophin and β_1 -syntrophin produced only moderate increases in agonist maximal responses.

This result was particularly surprising. One would expect that as receptor binding site density increases, agonist efficacy should increase as well. Thus, we felt compelled to perform a more thorough analysis to confirm this perplexing finding. First, we repeated this experiment using the same cells, except we used ERK1/2 phosphorylation as a functional output instead of IP production (Fig. 3B). Briefly, cells were cultured on 96 well plates, serum starved for 24 h and stimulated with increasing concentrations of phenylephrine for 10 min. Cells were probed for total and phospho-ERK and imaged using LiCor Odyssey. In agreement with our IP production experiments, phenylephrine maximal responses were significantly greater in β_2 -syntrophin expressing cells compared to α - and β_1 -syntrophin expressing cells, indicating this effect occurs regardless of the functional output measured.

As an additional test of this intriguing experimental result, we created concentration–response curves for full (norepinephrine, epinephrine) and partial (naphazoline, (+/–) *p*-octopamine, (+/–) *p*-synephrine and methoxamine) α_1 -AR agonists. If β_2 -syntrophin selectively increases α_{1D} -AR signal transduction coupling, we expected the efficacy of full and partial agonists would be significantly greater in β_2 -syntrophin expressing cells than in α - or β_1 -syntrophin expressing cells. Data are compiled in Table 3 and representative concentration–response curves for norepinephrine (Fig. 4A), epinephrine (Fig. 4B), methoxamine (Fig. 4C) and naphazoline (Fig. 4D) are shown. Interestingly, potencies (EC₅₀) for each agonist were not significantly different between α -, β_1 - or β_2 -syntrophin expressing cells, and all drug potencies were comparable to previously reported values [16–19]. However, agonist intrinsic activities were significantly greater in α_{1D} -AR/ β_2 -syntrophin cells (Fig. 4, Table 3) compared to α_{1D} -AR/ α -syntrophin cells. Indeed, partial agonists naphazoline and methoxamine acted as full agonists in α_{1D} -AR/ β_2 -syntrophin expressing cells.

Taken together, the results of our study suggest syntrophin isoforms play selective roles in the α_{1D} -AR/DAPC signalosome. α -syntrophin increases α_{1D} -AR binding site density while β_2 -syntrophin enhances α_{1D} -AR coupling to downstream signaling effectors. These experimental results may explain why α_{1D} -AR control of blood pressure is abrogated in double α/β_2 -syntrophinKO mice [5], as our findings suggest both α - and β_2 -syntrophin are required to create a functional α_{1D} -AR signalosome. However, it remains unknown precisely how α_{1D} -ARs, syntrophins and associated proteins utrophin, dystrobrevin, α -catulin are organized in the plasma membrane. We previously demonstrated α_{1D} -ARs form

homodimers *in vitro* and *in vivo* [5], thus we propose each protomer of the α_{1D} -AR homodimer associates with a different syntrophin isoform. Protomer 1 may associate with α -syntrophin to increase α_{1D} -AR homodimer binding site density, while Protomer 2 associates with β_2 -syntrophin to facilitate signal transduction. But how does each syntrophin isoform perform a selective function in the signalosome? One potential explanation is different syntrophin isoforms may selectively associate with unique interacting proteins. For example, we previously discovered in cell-based proteomic screens that β_2 -syntrophin selectively associates with the peripheral plasma membrane protein CASK (a.k.a. CAMGUK protein 2, calcium/calmodulin-dependent serine protein kinase 3, membrane associate guanylate kinase 2), which is a multidomain scaffolding protein that could potentially recruit in various signaling molecules to the complex [8]. Simultaneously, α -syntrophin could link α_{1D} -ARs to the cellular cytoskeleton through the DAPC and ensure proper folding and localization within the plasma membrane. Further studies will be required to validate these speculations.

In conclusion, our studies suggest the α_{1D} -AR/DAPC signalosome is a highly complex and tightly regulated GPCR signaling network, and that syntrophin isoforms play important and selective roles within the signalosome. Interestingly, 25 human GPCRs contain analogous Type-I PDZ binding motifs in their C-terminal domains, so this novel GPCR complex may represent a commonly used apparatus for global GPCR signaling.

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Abbreviations

AR	adrenergic receptor
DAPC	dystrophin-associated protein complex
ERK	extracellular signal regulated kinase
GPCR	G-protein coupled receptor
HEK	human embryonic kidney
KO	knock-out
PDZ	PSD95/DlgA/Zo-1

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Fig. 1.

Effect of syntrophin isoforms on α_{1D} -AR ligand affinity. Assays were performed using HEK293 cells stably expressing α_{1D} -ARs and α -syntrophin (■), β_1 -syntrophin (□) or β_2 -syntrophin (△). Representative competition binding assays are shown for the α_{1D} -AR antagonist BMY 7378 (A), the α_{1A} -AR antagonist 5-methylurapidil (B) and the nonselective α_1 -AR antagonist terazosin. (C) K_i values were calculated using non-linear regression and are shown in Table 1. Data were normalized to a percent [3 H]-prazosin bound. Data points are the mean $\pm$ SEM from 2–3 experiments performed in triplicate.

Fig. 2.

Effect of syntrophin isoforms on α_{1D} -AR binding site density. Assays were performed using HEK293 cells stably expressing α_{1D} -ARs alone (■) or co-expressed with α -syntrophin (□), β_1 -syntrophin (△) or β_2 -syntrophin (*). Binding site densities were measured using [3 H]-prazosin saturation radioligand binding assays. Non-specific binding was calculated as [3 H]-prazosin binding with 10 μ M phentolamine. B_{max} and K_D values were calculated using GraphPad Prism software and are reported in Table 2. Data points are the mean $\pm$ SEM from 2–3 experiments performed in triplicate.

Fig. 3. Effect of syntrophin isoforms on α_{1D} -AR signal transduction. (A) PI hydrolysis and (B) ERK1/2 activation assays were performed using HEK293 cells stably expressing α_{1D} -ARs alone ($\blacksquare$) or co-expressed with α -syntrophin ($\square$), β_1 -syntrophin ($\triangle$) or β_2 -syntrophin ($*$). Cells were stimulated with increasing concentrations of the α_1 -AR selective agonist phenylephrine (PE) for 10 min. Responses are reported as fold activity over basal. PE potencies and intrinsic activities were calculated using GraphPad Prism software. Data points are the mean $\pm$ SEM from 2–3 experiments performed in triplicate.

Fig. 4.

Effect of syntrophin isoforms on α_1 -AR full and partial agonist efficacy. Assays were performed using HEK293 cells stably expressing α_{1D} -ARs and α -syntrophin (■), β_1 -syntrophin (□) or β_2 -syntrophin (△). Representative concentration–response curves for PI hydrolysis are shown for the full agonists (A) norepinephrine, NE and (B) epinephrine, E and the partial agonists (C) methoxamine, MX and (D) naphazoline, NP. Responses are reported as fold activity over basal. Agonist potencies and intrinsic activities were calculated using GraphPad Prism software and are reported in Table 3. Data points are the mean $\pm$ SEM from 2–3 experiments performed in triplicate.

Table 1

Equilibrium dissociation constants for α_1 -AR antagonists at the α_{1D} -AR subtype. Data are expressed as pK_I values (M) and are the mean $\pm$ SEM of 3 experiments performed in triplicate.

Antagonist	+ α -syntrophin	+ β_1 -syntrophin	+ β_2 -syntrophin
BMY 7378	8.04 $\pm$ 0.11	8.46 $\pm$ 0.08	9.18 $\pm$ 0.16
5-MU	7.56 $\pm$ 0.17	7.63 $\pm$ 0.17	7.44 $\pm$ 0.41
Terazosin	7.89 $\pm$ 0.11	8.27 $\pm$ 0.07	8.82 $\pm$ 0.14
Benoxathian	7.76 $\pm$ 0.07	8.41 $\pm$ 0.04	8.40 $\pm$ 0.23
Cyclazosin	8.83 $\pm$ 0.14	9.03 $\pm$ 0.05	8.23 $\pm$ 0.19
Niguldipine	5.95 $\pm$ 0.30	6.30 $\pm$ 0.12	6.31 $\pm$ 0.10
Doxazosin	8.26 $\pm$ 0.10	8.57 $\pm$ 0.06	9.02 $\pm$ 0.10
A-315456	7.25 $\pm$ 0.13	7.27 $\pm$ 0.08	7.42 $\pm$ 0.21

Table 2

Effect of syntrophin isoforms on α_{1D} -AR binding site density and signal transduction coupling. The α_{1A} -AR +/- α -syntrophin and α_{1D} -AR +/- α -syntrophin data were previously reported in (REF). Phenylephrine concentration-response curves for stimulating PI hydrolysis and ERK1/2 phosphorylation were generated and used to calculate potency (pEC₅₀) and maximal responses (normalized to maximal α_{1A} -AR responses). Data shown are means +/- SEM from 3 experiments performed in triplicate.

Cell line	PI Hydrolysis			ERK1/2 Phosphorylation		
	Bmax (fmol/mg)	K _d (nM)	pEC ₅₀ (M)	Max (%)	pEC ₅₀ (M)	Max (%)
α_{1A} -AR	674.9 ± 148.1	1.56 ± 0.62	6.11 ± 0.14	102.0 ± 5.3	6.27 ± 0.35	87.6 ± 9.7
+ α -syntrophin	541.7 ± 28.1	1.14 ± 0.12	6.27 ± 0.07	102 ± 2.7	7.00 ± 0.22	81.7 ± 5.5
α_{1D} -AR	26.6 ± 7.5	0.22 ± 0.21	7.31 ± 0.66	32.6 ± 5.8	5.72 ± 0.66	48.4 ± 11.0
+ α -syntrophin	285.2 ± 51.7	0.80 ± 0.33	6.44 ± 0.19	95.9 ± 5.71	8.16 ± 0.56	98.8 ± 12.9
+ β_1 -syntrophin	123.4 ± 13.6	0.87 ± 0.21	6.27 ± 1.08	94.6 ± 36.8	4.18 ± 2.36	67.6 ± 107.8
+ β_2 -syntrophin	138.4 ± 27.6	0.52 ± 0.27	6.89 ± 0.14	295.2 ± 12.7	7.41 ± 0.48	177.4 ± 21.6

Table 3

Effect of syntrophin isoforms on agonist potencies and maximal responses. Maximum responses were normalized to maximal norepinephrine response for each α_{1D} -AR/syntrophin data set.

Agonist	+ α -syntrophin		+ β 1-syntrophin		+ β 2-syntrophin	
	pEC ₅₀ (M)	Max (%)	pEC ₅₀ (M)	Max (%)	pEC ₅₀ (M)	Max (%)
(+/-) Epinephrine	7.80 ± 0.08	82.5 ± 2.4	7.86 ± 0.11	132.1 ± 5.6 ^{***}	7.45 ± 0.22	126.4 ± 10.6 ^{***}
(+/-) Norepinephrine	7.78 ± 0.09	92.6 ± 3.2	7.55 ± 0.13	93.3 ± 4.3	8.00 ± 0.12	98.7 ± 4.4
(-) Phenylephrine	7.17 ± 0.11	73.8 ± 3.0	6.81 ± 0.12	92.5 ± 4.1 [*]	6.90 ± 0.12	109.6 ± 4.8 ^{***}
Naphazoline	8.00 ± 0.15	32.9 ± 1.6	7.24 ± 0.19	39.4 ± 2.5	7.35 ± 0.19	84.0 ± 5.3 ^{***}
(+/-) <i>p</i> -Octopamine	4.82 ± 0.31	73.0 ± 10.2	5.17 ± 0.16	104.7 ± 7.0 ^{***}	5.88 ± 0.23	38.7 ± 3.3 ^{***}
(+/-) <i>p</i> -Synephrine	5.53 ± 0.23	24.8 ± 2.1	5.38 ± 0.10	70.4 ± 2.8 ^{***}	5.65 ± 0.26	56.6 ± 5.4 ^{***}
Methoxamine	4.90 ± 0.11	59.9 ± 3.5	4.89 ± 0.18	66.7 ± 5.3	5.84 ± 0.14	93.6 ± 4.9 ^{***}

Agonist values obtained in β 1 and β 2-syntrophin cells were compared to those obtained in α -syntrophin cells using 2 way ANOVA and statistically significant differences are indicated by * ($p < 0.05$) and *** ($p < 0.001$). Data shown are the means $\pm$ SEM of 3 experiments performed in triplicate.