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Nuclear α1-Adrenergic Receptors Signal Activated ERK Localization to Caveolae in Adult Cardiac Myocytes

Casey D. Wright, **Quanhai Chen**, **Nichole L. Baye**, **Yuan Huang**, **Chastity L. Healy**, **Sivakanthan Kasinathan**, and **Timothy D. O'Connell**
Cardiovascular Research Center at Sanford Research/USD, Sioux Falls, SD

Abstract

We previously identified an α1A-adrenergic receptor-ERK survival signaling pathway in adult cardiac myocytes. Here, we investigated α1-AR subtype (α1A, α1B) localization and how their localization influences α1-AR signaling in cardiac myocytes. Using binding assays on myocyte subcellular fractions or a fluorescent α1-AR antagonist, we localized endogenousα1-ARs to the nucleus in wild-type adult cardiac myocytes. To clarify α1-subtype localization, we reconstituted α1-signaling in cultured α1A- and α1B-AR double knockout cardiac myocytes using α1-GFP fusion proteins. Similar to endogenous α1-ARs, α1A- and α1B-GFP co-localized with LAP2 at the nuclear membrane. α1-AR nuclear localization was confirmed *in vivo* using α1-AR-GFP transgenic mice. The α1-signaling partners Gαq and PLCβ1 also co-localized with α1-ARs only at the nuclear membrane. Furthermore, we observed rapid catecholamine uptake mediated by norepinephrine uptake-2 and found that α1-mediated activation of ERK was not inhibited by a membrane impermeant α1-blocker, suggesting α1-signaling is initiated at the nucleus. Contrary to prior studies, we did not observe α1-AR localization to caveolae, but we found that α1-AR signaling initiated at the nucleus led to activated ERK localized to caveolae. In summary, our results show that nuclear α1-ARs transduce signals to caveolae at the plasma membrane in cardiac myocytes.

Keywords

α1-adrenergic receptors; cardiac myocytes; ERK

INTRODUCTION

Cardiovascular disease is the leading killer in the United States, accounting for 1.4 million deaths a year. Five million Americans suffer from heart failure leading to 970,000 hospitalizations annually, a number which has tripled in the last 25 years.[1] In heart failure, increased activation of the sympathetic nervous system is correlated with pathophysiologic remodeling of the heart,[2] which led to the therapeutic use of β-adrenergic receptor (AR) antagonists in heart failure. However, the general conclusion that inhibition of catecholamine activation of ARs is beneficial in heart failure is disputed by clinical trials with α1-AR antagonists. The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) demonstrated that the α1-antagonist doxazosin increased the risk of heart failure by 80% and stroke by 26% leading to termination of the trial.[3, 4] Similar detrimental

Correspondence to: Timothy D. O'Connell, Cardiovascular Research Center, Sanford Research/USD, 1100 E. 21st St. Suite 700, Sioux Falls, SD 57105, Ph: 605-328-1300, Fax: 605-328-1301, oconnelt@sanfordhealth.org.

DISCLOSURES
None

effects were seen in the Vasodilator-Heart Failure Trials (V-HeFT), where prazosin was compared with other vasodilators for the prevention of death in heart failure.[5]

All three α1-AR subtypes (A, B, and D) are expressed in the heart,[6–9] however, cardiac myocytes only express the α1A and α1B.[9] Using α1A- and α1B-AR double knockout mice (α1ABKO), we demonstrated previously that α1-ARs are required for post-natal hypertrophy and adaptation to pathologic stress.[9, 10] In α1ABKO mice, we found that aortic constriction induced dilated cardiomyopathy that led to heart failure and death.[9] More recently, we identified an α1A-AR-ERK survival signaling pathway in adult cardiac myocytes,[11] and knockout of this α1A-ERK pathway could explain the maladaptive response to aortic constriction in α1ABKO mice. In summary, our data demonstrate that α1-ARs are protective in the heart, which agrees with clinical trials where α1-antagonists increased the incidence of heart failure.

Here, we examined the mechanisms of α1-AR survival signaling and expanded on our previous demonstration of α1-AR localization to the nucleus in adult cardiac myocytes.[11] To circumvent the lack of α1-AR subtype-specific antibodies and ligands, we developed a reconstitution system by expressing α1-AR-GFP fluorescent fusion proteins in culturedα1ABKO cardiac myocytes to examine α1-AR localization and survival signaling. With no endogenous α1-ARs, α1ABKO cardiac myocytes provide the ideal model for localization and signaling experiments. Earlier work in the mouse heart suggests that α1-ARs localize to the plasma membrane, possibly in caveolae, and that α1-AR signaling can be modified by caveolin-3.[12–15] These findings fit the classical “outside-in” model of G-protein coupled receptor (GPCR) localization and signaling because the immediate signaling partners (Gαq and phospholipase Cβ1) are also localized to caveolae.[16, 17] In contrast, our previous data usingα1-AR-GFP fluorescent fusion proteins suggested a nuclear localization for α1-ARs in adult cardiac myocytes.[11] Our identification of a GPCR (α1-AR) localized to the nucleus is not without precedence. Recently, functional endothelin-A, endothelin-B and β-adrenergic receptors were identified on the nucleus in adult cardiac myocytes.[18, 19]

Using our reconstitution system, we set out to clarify α1-AR localization and study the mechanisms behind α1-AR-ERK survival signaling in adult cardiac myocytes. In this report, we found that endogenous α1-ARs localized to the nucleus in wild-type adult mouse cardiac myocytes, which was confirmed *in vivo* using a cardiac-specific α1-AR transgenic mouse. Confocal microscopy and cellular fractionation demonstrated that the α1-AR subtypes, Gαq, and PLCβ1 localized to the nuclear membrane in adult cardiac myocytes. Furthermore, we found no evidence that either α1-AR subtype localized to the plasma membrane or caveolae. Finally, activation of functional α1-ARs on the nucleus led to accumulation of activated (phosphorylated) ERK in caveolae at the plasma membrane. Our results present a provocative new model by demonstrating that α1-AR signaling initiated at the nucleus results in activated ERK localization in caveolae at the plasma membrane.

MATERIALS AND METHODS

The procedures for generating adenoviral constructs,[11] culture of adult mouse cardiac myocytes,[10, 11] localization of α1-ARs by confocal microscopy,[11] isolation[18, 19] and enrichment[20] of nuclei, and measurement of α1-AR expression[9] were described elsewhere. Methods describing the generation of α1-AR-GFP transgenic mice, catecholamine uptake assay, treatment of cardiac myocytes with CGP-12177A, and detection of the extraneuronal monoamine transporter (EMT/OCT3) are contained in the supplemental methods. The use of animals in this study conformed to the PHS Guide for Care and Use of Laboratory Animals and was approved by The University of South Dakota Institutional Animal Care and Use Committee.

RESULTS

Endogenous α1-adrenergic receptors localize to the nucleus in WT adult mouse cardiac myocytes

Here, we set out to determine α1-AR subtype localization in adult cardiac myocytes. To examine α1-AR subcellular distribution, we isolated membrane, cytosolic and nuclear fractions from freshly isolated wild-type (WT) adult mouse cardiac myocytes and measuredα1-AR binding with ^{3}H-prazosin. Membrane, cytosolic, and nuclear fractions were validated by Western blots for caveolin-3, GAPDH, and LAP2 respectively (Figure 1a). Binding assays with ^{3}H-prazosin indicated that 80% of total α1-AR binding was detected in the nuclear fraction. Binding detected in the membrane fraction was likely due to α1-ARs localized to endoplasmic reticulum, as SERCA2 was detected in the membrane fraction (Figure 1a). To verify this finding, we examined endogenous α1-AR localization in cultured WT adult cardiac myocytes by labeling α1-ARs with a fluorescent, subtype non-selective α1-AR antagonist, BODIPY-prazosin, which overcomes the lack of subtype-specific α1-AR antibodies. BODIPY-prazosin labeling revealed that endogenous α1-ARs were concentrated around the nucleus but not on the plasma membrane in WT cardiac myocytes (Figure 1b). To verify that α1-ARs were expressed on the nucleus in cardiac myocytes, isolated nuclei from WT adult cardiac myocytes were also labeled with BODIPY-prazosin, and a strong signal was detected on the nuclear membrane (Figure 1B insets). In summary, these data suggest that endogenous α1-ARs localize primarily to the nucleus in WT adult cardiac myocytes.

The α1A and α1B subtypes co-localize with the nuclear membrane protein LAP2 in adult mouse cardiac myocytes

To test whether the α1A- and α1B-subtypes localize to the nucleus, we assessed co-localization of both α1-subtypes with the inner nuclear matrix protein LAP2. Again, to overcome the lack of subtype-specific antibodies, we expressed α1-fluorescent fusion proteins (α1-GFP) in cultured α1ABKO adult cardiac myocytes, which lack endogenous α1-ARs.[11] In this reconstitution system, the α1-GFP fusion proteins are expressed at only 2.5–3 fold over endogenous α1-AR levels.[11] Confocal microscopy revealed an intense GFP signal for both the α1A and α1B, and as expected, an intense fluorescent signal (red) for LAP2 on the nuclear membrane (Figure 2a). Using Imaris image analysis software, we demonstrated that both α1-subtypes co-localize with the nuclear matrix protein LAP2 (Figure 2a, yellow).

To confirm the confocal microscopy results, cellular fractions were isolated from cultured α1ABKO adult cardiac myocytes expressing the α1-GFP fusion proteins. Membrane, cytosolic, and nuclear fractions were validated by Western blots for caveolin-3, GAPDH, and LAP2 respectively (Figure 2b). As expected, both the α1A-GFP and α1B-GFP were detected predominantly in the isolated nuclear fraction (Figure 2b). In summary, the results obtained in our reconstitution system by confocal microscopy and subcellular fractionation demonstrated that the α1A- and α1B-subtypes were expressed in the nuclear membrane and confirmed the nuclear localization of endogenous α1-ARs in WT adult cardiac myocytes.

Prazosin induces α1-adrenergic receptors to move off the nuclear membrane in adult mouse cardiac myocytes

In cultured WT adult cardiac myocytes, BODIPY-prazosin labeling also identified a small population of non-nuclear α1-ARs (Figure 1b). We hypothesized that this was due to long-term incubation with BODIPY-prazosin, which induces β-Arrestin-dependent receptor internalization (movement off the membrane).[21] To test this, we expressed the α1-GFP fusion proteins in cultured α1ABKO cardiac myocytes and examined the α1-subtype localization throughout a 16 hr treatment with unlabeled prazosin. Indeed, prazosin induced translocation of both the α1A-GFP and α1B-GFP off the nucleus, whereas vehicle treatment had no effect

(Figure 3). This effect was seen at 2 hr and was consistent throughout treatment for the α1A-GFP (Figure 3, time course). In summary, the non-nuclear α1-AR population observed with BODIPY-prazosin staining is likely due to α1-AR translocation off the nuclear membrane, possibly by receptor desensitization, rather than a sub-population of non-nuclear α1-ARs.

α1-Adrenergic receptors localize to the nucleus in adult mouse cardiac myocytes in vivo

To determine if the nuclear α1-AR localization we observed in cultured adult cardiac myocytes was similar *in vivo*, we generated cardiac-specific α1A-GFP transgenic mice and used an antibody against GFP to identify α1A-subtype localization in whole heart sections. Heart sections from the α1A-GFP transgenic mice stained for GFP, identified the α1A-subtype at the nucleus (Figure 4), which parallels results from our culture model. Saturation binding analysis indicated that the α1A-GFP was overexpressed seven-fold in the heart (Online Figure Ia). Functionally, α1A-GFP overexpression increased α1-mediated phosphorylation of ERK, as determined by Western blot (Online Figure Ib). However, we did not detect a hypercontractile phenotype, as previously reported in α1A-Tg mice with 170-fold overexpression, nor did we detect a hypertrophic phenotype, as observed with overexpression of the α1B-subtype (data not shown).[12, 22, 23]

The α1A and α1B subtypes co-localize with Gαq and Phospholipase Cβ1 (PLCβ1) in the nuclear membrane in adult mouse cardiac myocytes

If functional α1-ARs are expressed on the nuclear membrane in adult cardiac myocytes, then the α1-signaling partners, Gαq and PLCβ1, should co-localize to the nucleus as well. To test this, we used immunocytochemistry to determine if Gαq and PLCβ1 were also expressed on the nuclear membrane. In cultured α1ABKO cardiac myocytes expressing α1A-GFP or α1B-GFP, we found that both Gαq and PLCβ1 localized to the nuclear membrane, as well as the plasma membrane (Figures 5a and 5b, respectively). However, image analysis revealed that both Gαq and PLCβ1 co-localized with the α1-subtypes only at the nuclear membrane.

To confirm the immunocytochemical analyses performed above, cellular fractionation and Western blot analysis were performed on freshly isolated WT adult mouse cardiac myocytes (Figure 5c). Western blots for caveolin-3, GAPDH, and LAP2 validated the purity of the membrane, cytosolic, and nuclear fractions (Figure 5c). Blotting the WT cell fractions for Gαq and PLCβ1 detected both signaling molecules in the nuclear fraction (Figure 5c). In summary, Gαq and PLCβ1 co-localized with both α1-AR subtypes only in the nuclear membrane (defined by LAP2 staining), which supports the hypothesis that α1-ARs could signal at the nucleus in adult cardiac myocytes.

α1-Adrenergic receptors do not localize to caveolae in adult mouse cardiac myocytes

The co-localization of the α1-AR subtypes, Gαq, and PLCβ1 to the nuclear membrane suggests that α1-ARs signal from the nucleus not the plasma membrane. However, previous research suggested that α1-ARs localize to caveolae in rats[17] and adult mice.[12] Furthermore, others demonstrated that increasing caveolin-3 expression inhibits α1-ERK signaling in cardiac myocytes, whereas knocking out caveolin-3 leads to hyperactivation of ERK signaling.[14, 15] Here, we immuno-labeled caveolin-3 to determine if the α1-AR subtypes localized to caveolae. In cultured α1ABKO cardiac myocytes expressing the α1-GFP fusion proteins, caveolin-3 localized to the plasma membrane and t-tubules whereas both α1-AR subtypes remained on the nucleus (Figure 6). The failure to observe caveolin-3 co-localization with either α1-AR subtype provides evidence against a direct interaction of α1-ARs with caveolin-3 in adult cardiac myocytes. These results also suggest that the modulation of α1-AR signaling by caveolin-3 likely occurs with signaling molecules downstream of the receptor itself.

Catecholamine uptake and activation of nuclear α1-adrenergic receptors in adult mouse cardiac myocytes

To activate nuclear α1-ARs, norepinephrine or other catecholamines, must enter the cardiac myocyte, a process known as norepinephrine-uptake-2,[24] which is facilitated by extraneuronal monoamine transporter (EMT/OCT3).[25] To measure catecholamine uptake in WT cardiac myocytes, we used a fluorescent catecholamine analog that fluoresces only when transported inside a cell. We found that catecholamine uptake occurred almost immediately, peaked at 30 min, and was antagonized by addition of norepinephrine 15 min prior to catecholamine uptake measurement, indicating specificity (Figure 7a).

To demonstrate the functionality of nuclear α1-ARs, we examined α1-AR mediated activation of ERK in cultured WT adult cardiac myocytes. Specifically, we compared the ability of CGP-12177A, a membrane impermeable α1-antagonist[26–29] and prazosin, which freely crosses the plasma membrane, to block phenylephrine (PE, α1-agonist) induced phosphorylation of ERK in cultured WT adult cardiac myocytes. Prazosin blocked PE-mediated activation of ERK, whereas CGP-12177A did not (Figure 7b), indicating that α1-AR mediated activation of ERK requires intracellular agonist and receptor.

In cultured WT adult cardiac myocytes, we detected EMT/OCT3 on both the plasma and nuclear membranes by immunocytochemistry (Figure 7c). Furthermore, we demonstrated that inhibiting EMT/OCT3 with corticosterone, an EMT/OCT3 antagonist, prevented α1-mediated activation of ERK (Figure 7c). Because our results indicate that α1-ARs, Gαq, and PLCβ1 localize to the nucleus, the results with CGP-12177A and corticosterone inhibition of EMT/OCT3 are best explained by α1-signaling at the nucleus.

Phosphorylated ERK localizes to caveolae at the plasma membrane in adult mouse cardiac myocytes

To investigate the mechanism of α1-mediated activation of ERK, we examined phosphorylated ERK localization following PE treatment. In cultured α1ABKO cardiac myocytes expressing α1A-GFP or α1B-GFP, PE led to phospho-ERK localization at the plasma membrane (Figure 8a). The α1A-GFP was more efficacious than α1B-GFP in activating ERK (Figure 8b), a difference we reported previously.[11, 30] Furthermore, in cardiac myocytes expressing the α1A-GFP, phenylephrine induced phospho-ERK plasma membrane localization within five minutes, which persisted for up to three hours (Figure 8c). The localization of phospho-ERK to the plasma membrane was surprising, since the receptor and its signaling molecules were localized to the nuclear membrane. However, short-term PE treatment, which activated ERK at the plasma membrane, did not translocate α1-ARs from the nucleus, suggesting α1-AR signaling was initiated at the nucleus, with a post-receptor signal translocated to the plasma membrane.

To determine if phosphorylated ERK localized to caveolae at the plasma membrane, we co-localized phospho-ERK and caveolin-3 following α1-AR stimulation in cultured α1ABKO cardiac myocytes expressing the α1A-GFP (Figure 8d). Following PE treatment, phospho-ERK and caveolin-3 co-localized at the plasma membrane. To verify the caveolar localization of phospho-ERK, we disrupted the assembly of caveolae using filipin.[31] Increasing concentrations of filipin reduced the co-localization of phospho-ERK and caveolin-3, suggesting an interaction between phospho-ERK and caveolin-3 (Figure 8d). A putative caveolin-binding domain is present in ERK and previous research has shown an interaction between ERK and caveolins,[32–34] but we could not confirm a direct interaction between ERK and caveolin-3 by co-immunoprecipitation. However, our results demonstrating a loss of phospho-ERK and caveolin-3 co-localization following filipin treatment indicates that ERK likely localizes to caveolae, possibly via some intermediate binding partner. Furthermore,

although α1-ARs do not localize to caveolae (Figure 6), α1-AR signaling could still be modified indirectly by caveolin-3 at the plasma membrane.[14, 15] In summary, our data suggest a novel model for α1-AR function in cardiac myocytes, where activation of nuclear α1-ARs leads to activated ERK localized to caveolae at the plasma membrane.

DISCUSSION

Here, we examined the localization of both the α1A- and α1B-subtypes and the mechanisms regulating α1A-AR-ERK survival signaling in adult cardiac myocytes. Our data indicate that both the α1A- and α1B-subtypes localize to and signal at the nuclear membrane. Furthermore, our data suggest that α1-signal transduction from the nucleus to the plasma membrane results in activated ERK localization to caveolae in adult cardiac myocytes. The following lines of evidence support this novel paradigm for α1-AR signaling in cardiac myocytes.

Using binding assays on subcellular fractions and a fluorescent α1-antagonist, we localized endogenous α1-ARs to the nucleus in WT adult cardiac myocytes, which was confirmed using α1-GFP fusion proteins in cultured α1ABKO cardiac myocytes and *in vivo* with α1-AR-GFP transgenic mice (Figures 1–4). Furthermore, we co-localized the α1-signaling partners Gαq and PLCβ1 with both α1-AR subtypes in the nuclear membrane (Figure 5), suggesting the possibility of α1-signaling at the nucleus. We failed to detect functional α1-ARs at the plasma membrane using confocal microscopy or functional studies examining α1-AR mediated activation of ERK (Figure 6, 7b). However, activation of nuclear α1-ARs led to accumulation of activated (phosphorylated) ERK at the plasma membrane in adult cardiac myocytes, while the receptor remained at the nucleus (Figures 8a). Finally, we demonstrated that phosphorylated ERK localizes to caveolae at the plasma membrane, which might provide a mechanism for caveolin-3 regulation of α1-AR signaling (Figure 8d).

Previously, others hypothesized that α1-ARs are expressed in caveolae at the plasma membrane in cardiac myocytes.[16, 17, 35] These reports relied on isolation of caveolar membrane fractions, followed by immunoblots to detect α1-signaling partners and functional examination of inositol phosphate generation. However, some of these reports acknowledged the failure to identify α1-ARs in caveolae, possibly due to basic (pH 11) conditions used to isolate the caveolar membrane fractions.[16, 35] Alternatively, our data indicated that α1-ARs are expressed on the nuclear membrane in cardiac myocytes. The use of BODIPY-prazosin andα1-GFP fusion proteins in our reconstitution system avoided the technical challenges of isolating caveolar membrane fractions. Prior research also demonstrated that overexpression of caveolin-3 suppressedα1-AR signaling,[15] and caveolin-3 knockout increased activation of ERK.[14] In contrast to others,[12, 17] we demonstrated that these effects were probably not due to a direct interaction of α1-ARs and caveolin-3. However, our finding that activated ERK localized to caveolae following α1-AR stimulation provides a mechanism where caveolin-3 might regulate α1-AR signaling. Earlier studies in cardiac-specific α1A-AR transgenic mice suggested that α1-ARs are expressed on the plasma membrane in cardiac myocytes *in vivo* based on immunohistochemical detection of the α1A-subtype in ventricular tissue sections.[12] However, we observed α1A localization to the nucleus in our α1A-GFP Tg mouse. This discrepancy might be due to the 170-fold level of overexpression examined in prior reports.[12] In summary, our results, both *in vivo* and *in vitro*, indicate that α1-ARs localize to the nuclear membrane in adult cardiac myocytes.

Nuclear localization of GPCRs in adult cardiac myocytes is not unique to α1-ARs. Functional endothelin-A and B receptors and β-ARs, as well as their cognate signaling proteins are also localized to the nuclear membrane of adult cardiac myocytes.[18, 19] Both of these reports directly demonstrated the functionality of their respective receptors on isolated nuclei, detecting a calcium transient in response to endothelin treatment[18] and cAMP accumulation in response

to isoproterenol treatment.[19] Here, we localized α1-ARs, Gαq, and PLCβ1 to the nuclear membrane and our experiments examining α1-mediated ERK activation indicate that α1-AR signaling was initiated at the nucleus in adult cardiac myocytes.

If α1-ARs localize to and activate signaling at the nucleus, then α1-agonists must enter the myocyte to initiate signaling. Norepinephrine entry into cardiac myocytes is facilitated by norepinephrine-uptake-2,[24] which is mediated by extraneuronal monoamine transporter (EMT/OCT3).[25] A previous report demonstrated that ^{3}H-norepinephrine is taken-up by neonatal rat cardiac myocytes and accumulates in the nucleus.[36] We confirmed catecholamines are taken-up in adult cardiac myocytes, and detectable inside the cell within 5 min (Figure 7a). In support of the results that demonstrate agonists like norepinephrine enter the myocyte to activate α1-ARs, we found that CGP-12177A, a membrane impermeable α1-AR antagonist, was unable to block phenylephrine-induced activation of ERK in cardiac myocytes, whereas prazosin did (Figure 7b). Finally, we also identified EMT/OCT3 on both the plasma and nuclear membranes and confirmed its role in catecholamine uptake in adult cardiac myocytes (Figure 7c).

In total, our data suggest a new model for α1-AR survival signaling. We propose that ligand enters the cardiac myocyte, binds to α1-ARs and activates α1-signaling at the nucleus, which is transduced from the nucleus to the plasma membrane, causing activated ERK localization to caveolae and protection from cell death. This novel α1-signaling model does not fit the classical “outside-in” GPCR signaling model or the “caveolae signaling hypothesis” as described previously.[33] However, some details of this new model remain to be determined. First, the mechanism regulating α1-signal transduction from the nucleus to the plasma membrane is unknown, but could involve protein kinase C, which is activated by α1-ARs, translocates to caveolae when activated,[37] and could lead to ERK phosphorylation. Second, potential downstream targets of α1-ERK signaling in cardiac myocytes are unidentified. Previous work in neonatal cardiac myocytes suggested that targets could include the survival/transcription factor GATA4 and the Bcl-2 family member Bad.[38, 39] However, we recently demonstrated that α1-signaling does not activate GATA4 and that GATA4 was not required for α1-mediated survival signaling in adult cardiac myocytes.[40]

In summary, we found that the α1A and α1B subtypes localized to and activated signaling at the nuclear membrane in adult cardiac myocytes. Activation of these nuclear α1-ARs led to accumulation of activated ERK at the plasma membrane where caveolin-3 might regulate downstream α1-AR signaling. These results present a provocative new model for α1-AR signaling, where nuclear α1-ARs signal to the plasma membrane. This unique α1-AR-ERK survival-signaling pathway challenges the classic model of α1-AR localization and signaling in adult cardiac myocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

α1A-AR	α1A-adrenergic receptor
α1B-AR	α1B-adrenergic receptor
α1A-GFP	α1A-adrenergic receptor-GFP fusion
α1B-GFP	α1B-adrenergic receptor-GFP fusion
α1ABKO	α1A andα1B adrenergic receptor double knockout mice
PE	phenylephrine

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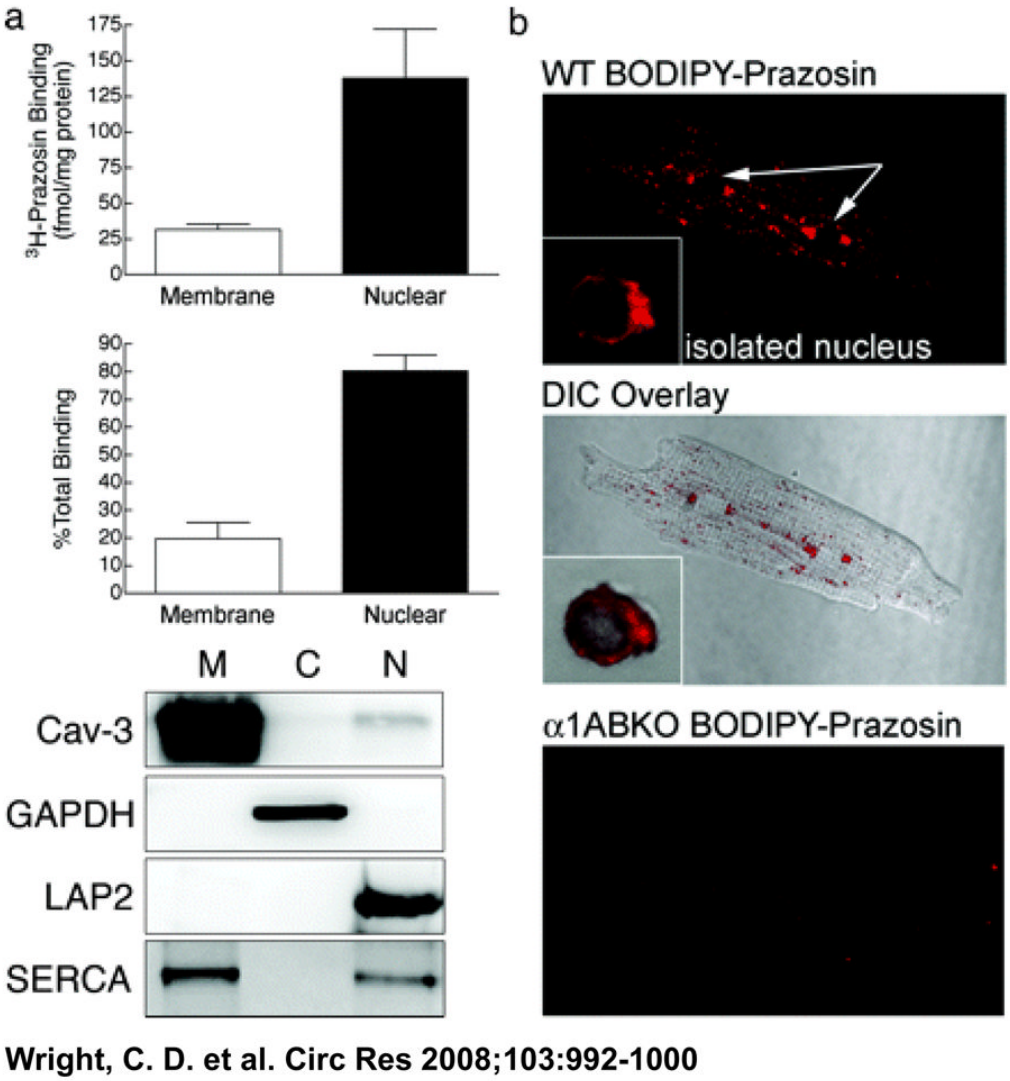

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Figure 1. Endogenous α1-adrenergic receptors localize to the nucleus in WT adult mouse cardiac myocytes

(**a**) WT myocytes were fractionated by homogenization and ultracentrifugation. Membrane (M), cytosolic (C), and nuclear (N) fractions (30 μg) were validated by Western blots for caveolin-3, GAPDH, LAP2, and SERCA. α1-AR levels in WT membrane and nuclear fractions were determined by a single-point, maximum-concentration, binding assay with ^{3}H-prazosin (1.2 nM), where 10 mM phentolamine (RBI, Natick, MA) defined non-specific binding. (**b**) WT andα1ABKO adult mouse cardiac myocytes (AMMC) were cultured for 24 hr then incubated with 50 nM BODIPY-prazosin (non-specific α1-AR antagonist) for 16 hr, fixed with paraformaldehyde and confocal images captured. Myocyte nuclei are identified by the white arrows. Final magnification=600×. ***Inset:*** Isolated nuclei were incubated with 50 nM BODIPY-prazosin for 1 hr and confocal images captured. Final magnification=1,200×.

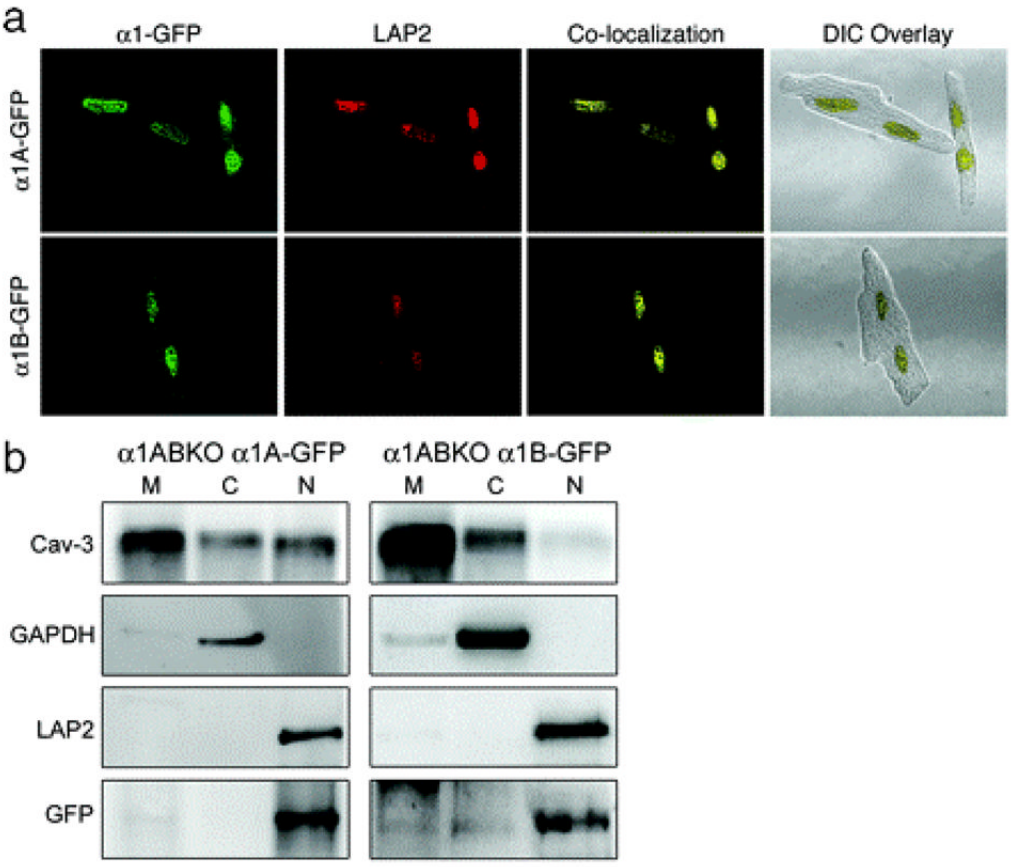

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Figure 2. The α1A and α1B subtypes co-localize with the nuclear membrane protein LAP2 in adult mouse cardiac myocytes

(**a**) Cultured α1ABKO cardiac myocytes were infected with adenoviruses expressing the α1A-GFP (1,000 MOI) or α1B-GFP (3,000 MOI). After 40 hr, myocytes were fixed with 4% paraformaldehyde and stained with an antibody against the nuclear membrane marker LAP2, and a Texas red-conjugated secondary antibody. Fluorescent and transmitted light images were captured by confocal microscopy and co-localization was determined using Imaris software. Final magnification=600×. (**b**) Cultured α1ABKO cardiac myocytes were infected as above and fractionated as in Figure 1. Western blots were performed to detect the α1-subtypes using an antibody to GFP.

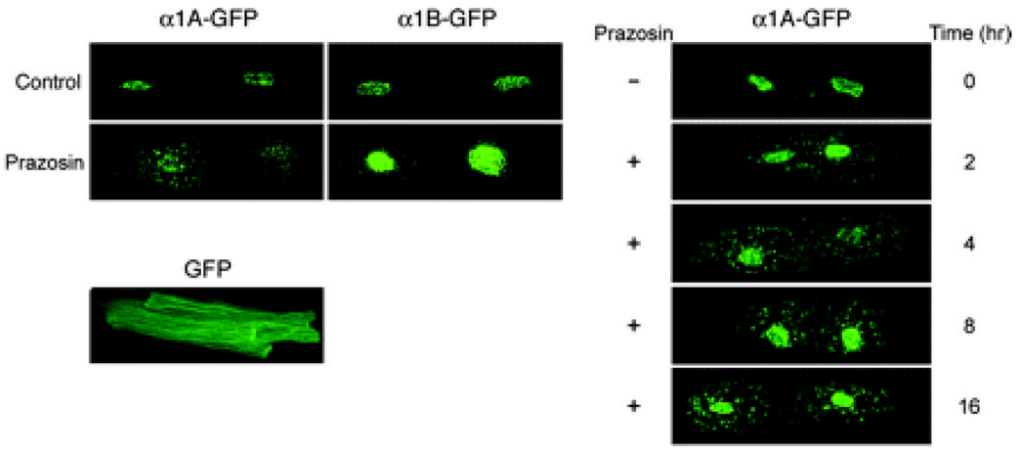

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Figure 3. Prazosin induces α1-adrenergic receptors to move off the nuclear membrane in adult mouse cardiac myocytes

Cultured α1ABKO cardiac myocytes were infected as in Figure 2. After 24 hr, myocytes were treated with prazosin (50 nM) or vehicle (control) for 16 hr then fixed with paraformaldehyde and confocal images captured. Final magnification=600×.

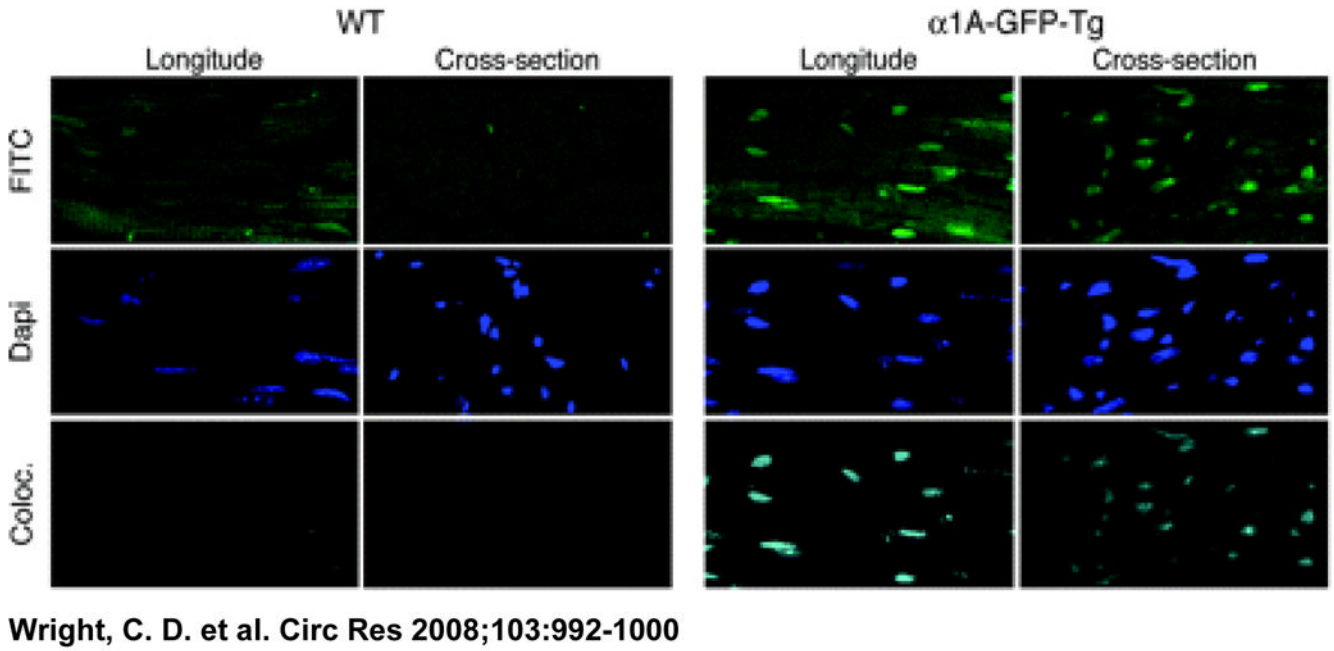

Figure 4. α1-adrenergic receptors localize to the nucleus in adult mouse cardiac myocytes *in vivo*
Sagittal sections from hearts of α1A-GFP transgenic mice (α1A-GFP Tg) or WT mice were stained with anti-GFP antibody (Santa Cruz) to detect α1A-GFP and visualized using the Mouse-on-Mouse Kit with a FITC-conjugated secondary antibody (Vector Laboratories).

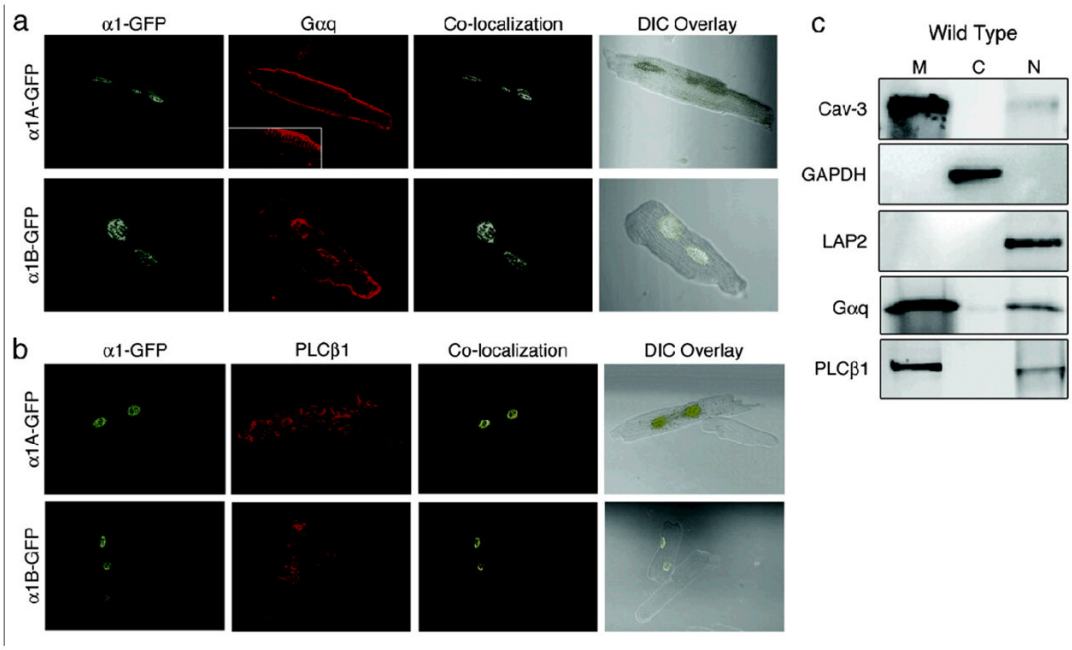

Figure 5. The α1A and α1B subtypes co-localize with Gαq and Phospholipase Cβ1 (PLCβ1) in the nuclear membrane in adult mouse cardiac myocytes

α1ABKO cardiac myocytes were cultured, infected, and fixed as in figure 2 then stained with an antibody against **(a)** Gαq or **(b)** PLCβ1 and a Texas red conjugated secondary antibody and confocal images captured and prepared as in figure 2. Final magnification=600×. **(c)** Western blots were performed on WT cardiac myocyte cell fractions to detect Gαq and PLCβ1.

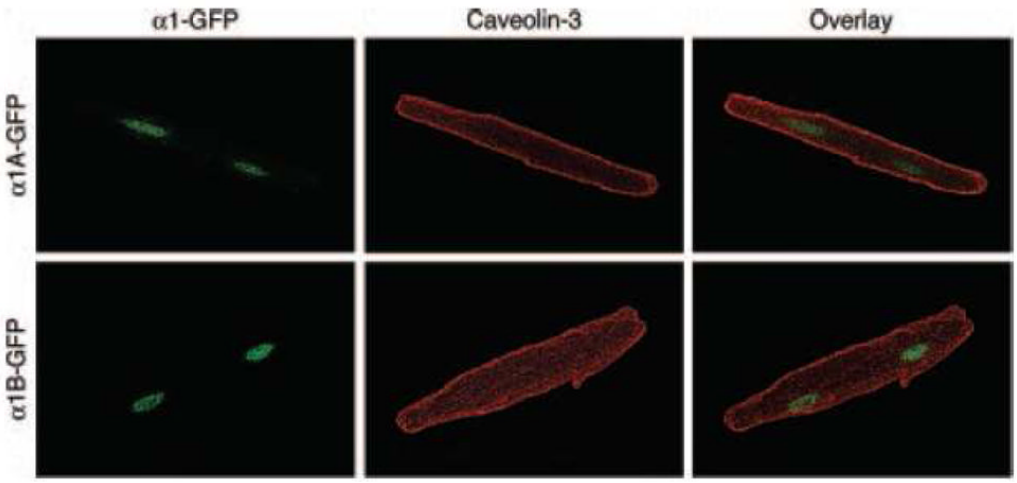

Figure 6. α1-Adrenergic receptors do not localize to caveolae in adult mouse cardiac myocytes

Cultured α1ABKO cardiac myocytes were infected as in figure 2, fixed with 4% paraformaldehyde, and stained with an antibody against caveolin-3 and an Alexa Fluor-594-conjugated secondary antibody. Fluorescent and transmitted light images were captured by confocal microscopy and co-localization determined using Imaris software. Final magnification=600×.

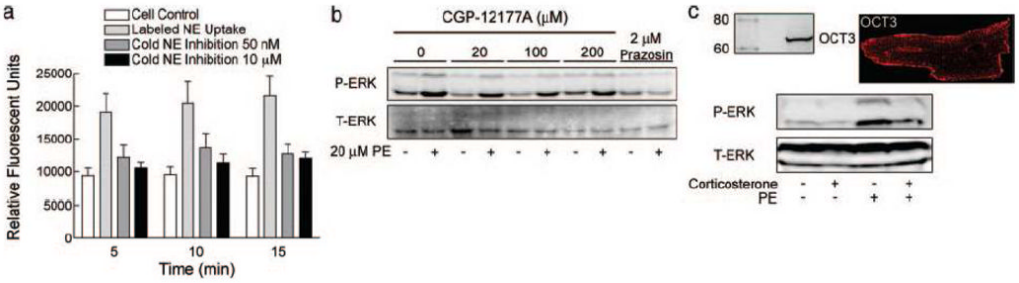

Figure 7. Catecholamine uptake and activation of nuclear α1-adrenergic receptors in adult mouse cardiac myocytes

(**a**) Catecholamine uptake assay (Molecular Devices, Sunnyvale, CA) was used to determine norepinephrine transport into WT cardiac myocytes. Specificity for norepinephrine uptake was confirmed by addition of norepinephrine (50 nM or 10 μM) 15 min prior to catecholamine uptake measurement. The results represent triplicate measurements from 4–5 myocyte isolations. (**b**) Cultured WT cardiac myocytes were treated with CGP-12177A (0–200 μM, membrane impermeable α1-AR antagonist) or prazosin for 15 min, and then with phenylephrine (20 μM, PE) for 15 min. Phospho- and total ERK levels were determined by Western blot. (**c**) Cultured WT cardiac myocytes were harvested for Western blot detection of OCT3 or fixed with 4% paraformaldehyde, and stained with an antibody against OCT3 (Santa Cruz) and an Alexa Fluor-594-conjugated secondary antibody. Fluorescent images were captured by confocal microscopy. Final magnification=600×. Cultured WT cardiac myocytes were treated with corticosterone (1 μM) for 15 min, then PE (20 μM) for 15 min. Phospho- and total ERK levels were determined by Western blot.

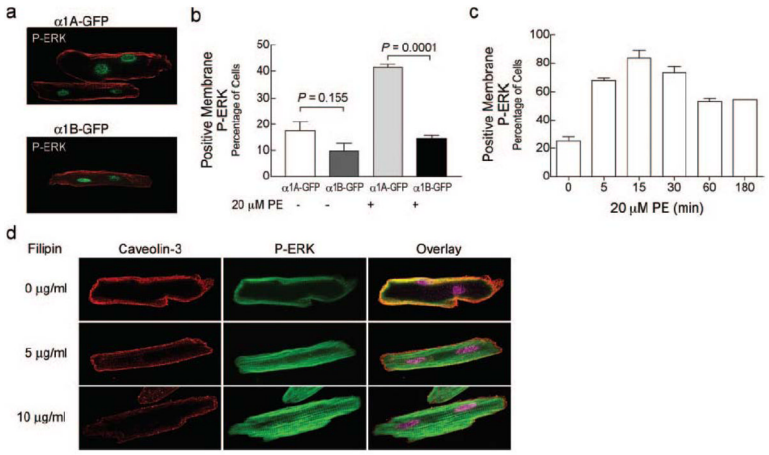

Figure 8. Phosphorylated ERK localizes to caveolae at the plasma membrane in adult mouse cardiac myocytes

(**a**) Cultured α1ABKO cardiac myocytes were infected as in Figure 2. After 40 hr, myocytes were treated with 20 μM phenylephrine (PE) for 15 min. Myocytes were fixed with 4% paraformaldehyde and stained with an antibody against P-ERK and a Texas red-conjugated secondary antibody. Fluorescent images were captured by confocal microscopy. Final magnification=600×. (**b, c**) The percentage of myocytes positive for P-ERK (n=100–124 myocytes/culture) was determined comparing (c) α1A-GFP and α1B-GFP (n=3) or (d) theα1A-GFP time course (n=2). Groups were compared by one-way ANOVA with a Tukey's post-test ($P<0.05$). (**d**) Isolated α1ABKO cardiac myocytes were cultured and infected as above then treated with filipin (5–10 μg/ml) or vehicle for 1 hr followed by 20 μM PE for 15 min. Myocytes were fixed and stained with antibodies against P-ERK and caveolin-3, and images were captured by confocal microscopy and pseudocolored using Imaris software (GFP pseudocolored magenta and P-ERK green). Final magnification=600×.