Adrenergic Receptors in Individual Ventricular Myocytes

The Beta-1 and Alpha-1B Are in All Cells, the Alpha-1A Is in a Subpopulation, and the Beta-2 and Beta-3 Are Mostly Absent

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Rationale: It is unknown whether every ventricular myocyte expresses all 5 of the cardiac adrenergic receptors (ARs), β1, β2, β3, α1A, and α1B. The β1 and β2 are thought to be the dominant myocyte ARs.

Objective: Quantify the 5 cardiac ARs in individual ventricular myocytes.

Methods and Results: We studied ventricular myocytes from wild-type mice, mice with α1A and α1B knockin reporters, and β1 and β2 knockout mice. Using individual isolated cells, we measured knockin reporters, mRNAs, signaling (phosphorylation of extracellular signal–regulated kinase and phospholamban), and contractile force. We found that the β1 and α1B were present in all myocytes. The α1A was present in 60%, with high levels in 20%. The β2 and β3 were detected in only ≈5% of myocytes, mostly in different cells. In intact heart, 30% of total β-ARs were β2 and 20% were β3, both mainly in nonmyocytes.

Conclusion: The dominant ventricular myocyte ARs present in all cells are the β1 and α1B. The β2 and β3 are mostly absent in myocytes but are abundant in nonmyocytes. The α1A is in just over half of cells, but only 20% have high levels. Four distinct myocyte AR phenotypes are defined: 30% of cells with β1 and α1B only; 60% that also have the α1A; and 5% that also have the β2 or β3. The results raise cautions in experimental design, such as receptor overexpression in myocytes that do not express the AR normally. The data suggest new paradigms in cardiac adrenergic signaling mechanisms. (Circ Res. 2017;120:1103-1115. DOI: 10.1161/CIRCRESAHA.117.310520.)

Key Words: adrenergic agents ▪ mice, knockout ▪ receptors, adrenergic ▪ receptors, adrenergic, beta ▪ myocytes, cardiac ▪ receptors, adrenergic, alpha-1

The heart has 5 main adrenergic receptors (ARs), β1, β2, β3, α1A, and α1B, plus a small number of α1D and α2 on vessels and nerves, which mediate the effects of the catecholamines norepinephrine (NE) and epinephrine (EPI). The β1 and β2 are considered the most important cardiac ARs, with a minor role for α1A and β3. 1-3 β-ARs control the rate and strength of cardiac contraction. The role of the α1B might be cardiac growth, 3 and the β2, β3, and α1A are each implicated in cardioprotection. 4 Current AR radioligand binding data in heart suggest β-AR dominance, comprising 90% β-ARs, present in an 8:2 ratio of β1:β2, and 10% α1-ARs, present in a 6:4 ratio of α1A:α1B. 3 However, few data exist on binding in isolated cardiac myocytes.

Models of adrenergic signaling in the heart do not consider whether all 5 receptors are actually present on all myocytes. One model is that ARs are distributed equally among cells, according to their respective levels in myocardial binding assays. Thus, investigations typically present grouped data for AR signaling in isolated myocytes, with no accounting of myocytes that have no or low receptor levels. Similarly, AR function is tested using forced expression by transgenic and virus approaches in all myocytes, without knowing whether these approaches mimic normal physiology. Expression of the 5 ARs on individual myocytes has never been studied.

Previously, we used an α1A-AR knockout (KO) reporter mouse, with bacterial β-galactosidase (bGal) replacing exactly

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the first coding exon, to show that α1A expression in the abdominal arteries was markedly heterogeneous, raising the question whether the same could be true for heart. Here, we studied all 5 ARs in individual cardiac myocytes. We used the α1A reporter mouse and a new reporter for the α1B ARs in individual cardiac myocytes. We measured in individual ventricular myocytes and nonmyocytes, including endothelial cells.

Catecholamines and their ARs have a crucial role in cardiac biology. Surprisingly, the ARs actually present in individual ventricular myocytes are not known. There are no valid AR antibodies, so we used AR expression (mRNAs, knockin reporters) and function (signaling, contraction) to show for the first time that the β1 and α1A are in all myocytes, the α1A is in a subpopulation, and the β2 and β3 are mostly absent in myocytes, but abundant in nonmyocytes. α1A levels varied up to 100-fold. These new data have important implications. Experimentally, for example, it is not physiological to overexpress an AR in myocytes that do not express that AR normally, and myocyte studies need to be aware that individual myocytes differ substantially in their AR composition. Biologically, the β1 and α1B are the intrinsic regulators of myocyte responsive effects, not the β1 and β2 as thought. The α1B might have a “braking” function on the β1. α1A contractile and protective effects in the intact heart are presumably mediated via some myocyte-to-myocyte communication. For the β2 and β3, the absence of downregulation in heart failure might reflect proliferation of nonmyocytes, and any cardioprotective effects of receptor agonists or blockers need to be interpreted relative to actions in nonmyocytes.

What Is Known?
• Adrenergic receptors (ARs) mediate the ubiquitous effects of the catecholamines norepinephrine and epinephrine on heart structure and function.
• The β1- and β2-ARs are considered to be the main cardiac myocyte ARs, with minor levels of the α1A, α1B, and β3.
• No study has identified cardiac ARs in individual ventricular myocytes.

What New Information Does This Article Contribute?
• We quantified expression and function of β- and α1-ARs in individual adult mouse ventricular myocytes.
• We found that all myocytes have the β1- and β1B-ARs.
• The α1A-AR is in a 60% myocyte subpopulation and at high levels in only 20% of cells.
• The β2- and β3-ARs are mostly absent in myocytes, but are abundant in nonmyocytes, including endothelial cells.

Nonstandard Abbreviations and Acronyms
• α-MyHC α-myosin heavy chain
• AKO α1A-AR knockout
• bGal β-galactosidase
• Cq quantification cycle
• NRVM neonatal rat ventricular myocyte
• pERK phosphorylated extracellular signal–regulated kinase
• PTX pertussis toxin
• pPLN phosphorylated phospholamban
• RT-qPCR reverse transcription quantitative real time polymerase chain reaction
• WT wild-type

Methods
Mice were primarily adult males in the C57BL/6J background. α1A-KO reporter mice have bGal replacing exactly the first coding exon. The Mouse Biology Program at the University of California, Davis, constructed α1B-KO mice with human placental alkaline phosphatase (hPLAP) replacing the first coding exon. β1/2-KO mice were from Jackson Laboratories (#003810) and in a mixed background (C57BL/6J, DBA/2, 129, FVB/N, CD-1); mice for study were obtained by backcross into C57Bl/6J and then intercrossing littermates for KO and WT controls. Mice in PKCδ (protein kinase C) experiments were in FVB/N129 mixed background. Adult mouse ventricular myocytes were isolated by perfusion with collagenase; right ventricle (RV), septum, and left ventricle (LV) were dissociated separately in some experiments. Sprague–Dawley neonatal rat ventricular myocytes (NRVMs) were isolated with trypsin. Adult mouse ventricular myocytes and NRVMs were cultured in serum-free medium.

Reporter gene staining was done in perfused heart or isolated myocytes, and stained hearts were used for paraffin sections or optical projection tomography. bGal enzyme activity in heart lysates was assayed with the Galacto-Star chemiluminescent reporter gene assay system. RNA from hearts was extracted with RNeasy Mini Kit, and α1A mRNA normalized to β-actin mRNA was quantified by reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) and the ΔΔCq method. Single myocytes were extracted and amplified using the Sigma WTA kit and used on NimbleGen Gene Expression Arrays to measure mRNAs for α1A, α1B, β2, and β3. β1- and α1A mRNAs were also quantified by RT-qPCR on single cells.

ERK1/2 (extracellular signal–regulated kinase 1/2) dually phosphorylated on tyrosine and threonine (pERK) was detected in cultured myocytes by immunoblot or immunocytochemistry with a rabbit monoclonal antibody (Cell Signaling #4370) and phospholamban (PLN) phosphorylated on serine 16 (pPLN), with a rabbit polyclonal antibody (Upstate #07–052). Fluorescence intensity in individual myocytes was quantified from 1 s digital images using Quantity One (Bio-Rad) and Image J (NIH IJ1.48v) software. Nuclear translocation of PKCδ in frozen sections of perfused heart was detected with a purified mouse monoclonal antibody (BD Transduction Laboratories #P36520-610397).

Isolated myocyte contraction was quantified by changes in sarcomere length using an IonOptix system.

Saturation radioligand binding in total membranes from isolated cardiac myocytes, nonmyocytes, and intact hearts used 3H-prazosin for α1-ARs and 125I-cyanopindolol (CYP) for β-ARs. The fraction of AR subtypes was determined using competition binding in WT and binding in KOs.

Results are reported as mean±SE. GraphPad Prism v5.0d was used to test for a normal distribution; to test for significant differences (P<0.05), using t test or 1-way ANOVA and Newman–Keuls post test.
for >2 groups; to do linear regression; and to analyze binding data and concentration–response curves.

**Results**

**Knockin Reporter Mice Show the α1A in a Myocyte Subset, the α1B in All Cells**

The knockin mice had bacterial bGal (α1A) or hPLAP (α1B) inserted exactly at the translational start sites, so that expression was controlled by all endogenous regulatory elements. Online Figure IA shows that bGal enzyme activity in AKO heart was highly correlated with α1A mRNA levels in WT heart from birth, when α1A mRNA was barely detectable, through adulthood ($r^2=0.94; P<0.001$), validating bGal as a surrogate for α1A transcription.

In the intact AKO heart, bGal was heterogeneous, with stain intensity from none to high, as visualized by conventional histochemistry or by optical projection tomography (Figure 1A; Online Movies). This heterogeneity was also seen in isolated myocytes; Figure 1A shows 5 cells, only 1 of which has blue dots of bGal. We verified that the staining protocol detected the maximum number of positive cells, and paraffin sections of AKO heart confirmed that stained and unstained myocytes were in intact tissue (Online Figure II). The number of bGal dots in isolated myocytes varied from none to high ($\geq 7$; Online Figure IB). Overall, we counted dots in ≈16000 cells from 33 hearts and heart regions and found that bGal was present in 61±1% of myocytes in all ventricular regions (LV and RV free walls, septum) and that bGal was absent in 39±1%; an average 18% of cells had high bGal levels ($\geq 7$ dots). Selection bias was not a confounder because identical numbers of myocytes were isolated from AKO and WT ventricles (AKO $2.1\pm 0.07\times 10^6$, n=33 hearts versus WT $2.1\pm 0.05\times 10^6$, n=103; $P=0.2$).

In the intact α1BKO heart, alkaline phosphatase (alk phos) stain, after heat inactivation of endogenous alk phos, was intense throughout the heart (Figure 1B). All KO myocytes were stained, versus none in WT (Figure 1B).

In summary, 61% of AKO myocytes in all LV and RV regions have bGal, an α1A transcription reporter, with 18% having high levels, and 100% of α1BKO cells have alk phos, an α1B reporter.

**α1 mRNA Levels in WT Myocytes Show the α1A in a Myocyte Subset, the α1B in All Cells**

To test α1A and α1B expression in WT myocytes, we measured mRNA levels in each of 50 cells using microarray. As shown in Figure 2, we considered a cell positive for an mRNA if the mean±SE mRNA level from the 3 to 6 probes on the array was greater than the background level defined by 5000 Figure IB). Overall, we counted dots in ≈16000 cells from 33 hearts and heart regions and found that bGal was present in 61±1% of myocytes in all ventricular regions (LV and RV free walls, septum) and that bGal was absent in 39±1%; an average 18% of cells had high bGal levels ($\geq 7$ dots). Selection bias was not a confounder because identical numbers of myocytes were isolated from AKO and WT ventricles (AKO $2.1\pm 0.07\times 10^6$, n=33 hearts versus WT $2.1\pm 0.05\times 10^6$, n=103; $P=0.2$).

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In summary, 61% of AKO myocytes in all LV and RV regions have bGal, an α1A transcription reporter, with 18% having high levels, and 100% of α1BKO cells have alk phos, an α1B reporter.
probes directed to nonsense sequences. By this criterion, 25 of 50 myocytes were positive for the α1A, whereas all cells had the α1B (Figure 2A). Nine of 50 cells had high α1A (18%). Single-cell qPCR confirmed this result, with 50% of 18 WT myocytes containing α1A mRNA and 22% having high levels (Figure 5). Interestingly, there was no correlation between levels of the α1A and α1B in individual myocytes (Figure 2B). All cells studied had α-myosin heavy chain (MyHC) much greater than β-MyHC, as expected (Figure 2C).

In summary, 50% of WT myocytes have α1A mRNA, with an average 20% having high levels, and 100% have α1B.

**An α1A Agonist Activates ERK in a Subset of Myocytes**

We could not use immunocytochemistry with α1-antibodies to test for α1A protein because commercial α1-antibodies are nonspecific for any α1-subtype or for total α1-ARs. Therefore, to test for α1A protein and for functional significance of reporter and mRNA expression in only 60% of cells, we used a signaling assay, activation of ERK, which is cardioprotective with α1A activation. We used the highly selective and potent α1A agonist, A61603, and quantified maximum ERK activation in individual cells using immunofluorescence with an antibody for dually phosphorylated ERK1/2.

Figure 3A shows an experiment in which 1 of 2 A61603-treated myocytes had diffuse cytoplasmic staining for pERK (green), and the other myocyte had no detectable pERK. Mean pERK fluorescence per cell was quantified by digital microscopy in ≈250 cells per group. Figure 3B is a representative experiment, with 62% of A61603-treated WT myocytes having positive pERK fluorescence, defined as greater than the median plus range of vehicle-treated cells; median was used because values were not distributed normally. In the same experiment, 93% of phorbol myristate acetate (PMA)–treated cells had pERK above this level, as a positive control, and none of A61603-treated AKO cells as a negative control. In the experiment shown, 19% of A61603-treated WT cells had pERK levels as high as the highest seen with PMA (Figure 3B), and overall 20±1% of A61603-treated cells had high pERK in 3 identical experiments. Figure 3C shows blinded microscopic counts of 200 to 250 myocytes, indicating that A61603 activates ERK in 62% of WT myocytes, with 20% activating ERK to the same high extent as seen with the PKC activator PMA.
or a negative inotropic effect, as observed previously.\textsuperscript{12} A61603 responders and A61603 nonresponders contracted equally with isoproterenol (ISO; Figure 8), showing that all of the cells were competent to contract with β-AR stimulation. A61603 was inactive in all AKO cells, confirming selectivity (Figure 4B), and AKO cells were competent to respond to phenylephrine (below). Combining WT cells with either a positive inotropic effect or a negative inotropic effect, 21% had a high response to A61603, defined as ≥240% change in contraction (Figure 4B).

To test for function of the α1B, we treated AKO myocytes, which have only the α1B,\textsuperscript{13} with the nonselective α1-adrenergic receptor knockout (AKO) hearts; bars are mean±SE, with each of 5 experiments with different WT and α1A-KO cells treated with A61603 or phorbol myristate acetate (PMA; 100 nmol/L) as a positive control. α1A-KO cells treated with A61603 are a negative control. Each dot is 1 cell. Percent values indicate the fraction of positive cells with fluorescence greater than the vehicle median+range (dotted line). C, The percent of myocytes positive for pERK increased by 5000 probes directed to nonsense sequences. Surprisingly, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels.

The α1A Mediates Contraction in a Subset of Myocytes, the α1B in all Cells

As a second end point for the presence and function of the α1A in individual myocytes, we tested activation of contraction by a maximum concentration of the selective agonist A61603 in isolated WT and AKO myocytes.\textsuperscript{11} Figure 4A shows typical raw tracings of sarcomere length over time, and Figure 4B has summary data for 61 cells from 20 hearts. A61603 changed contraction in 64% of 39 WT myocytes, defined as greater than the median±range of vehicle-treated cells. In different cells, A61603 stimulated a positive inotropic effect or a negative inotropic effect, as observed previously.\textsuperscript{12} A61603 responders and A61603 nonresponders contracted equally with isoproterenol (ISO; Figure 8), showing that all of the cells were competent to contract with β-AR stimulation. A61603 was inactive in all AKO cells, confirming selectivity (Figure 4B), and AKO cells were competent to respond to phenylephrine (below). Combining WT cells with either a positive inotropic effect or a negative inotropic effect, 21% had a high response to A61603, defined as ≥240% change in contraction (Figure 4B).

To test for function of the α1B, we treated AKO myocytes, which have only the α1B,\textsuperscript{13} with the nonselective α1-adrenergic receptor knockout (AKO) hearts; bars are mean±SE, with each of 5 experiments with different WT and α1A-KO cells treated with A61603 or phorbol myristate acetate (PMA; 100 nmol/L) as a positive control. α1A-KO cells treated with A61603 are a negative control. Each dot is 1 cell. Percent values indicate the fraction of positive cells with fluorescence greater than the vehicle median+range (dotted line). C, The percent of myocytes positive for pERK increased by 5000 probes directed to nonsense sequences. Surprisingly, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels. Also notable, the range of mRNA levels is simply a reflection of overall AR levels.
by this criterion, only 4 of 50 myocytes (8%) were positive for β2 or β3 mRNA (Figure 6A). Interestingly, plotting β2 and β3 mRNAs versus increasing α1A mRNA in the same cells showed that the 3 mRNAs were mostly in different cells (Figure 6B).

To test the unexpected absence of β2 and β3 mRNAs in myocytes by an alternate method, we did radioligand binding in heart and in isolated myocytes. To quantify the β-AR subtypes unambiguously, we compared WT with β1-KO and β1/2-double KO. In myocytes, all β-AR binding in WT cells was still present in β2-KO cells, whereas specific binding was undetectable in β1-KO and β1/2-KO myocytes (Figure 6C). In intact heart, in contrast, β3 binding was evident in β1/2-KO heart, and β2 binding could be calculated from values in the β1- and β1/2-KO hearts (Figure 6C). These data suggested that total β-ARs in intact heart were ≈50% β1, 30% β2, and 20% β3, indicating sizable populations of β2 and β3 in nonmyocytes, but no detectable β2 and β3 binding in myocytes.

To confirm that the β2 and β3 were present in nonmyocytes, we did RT-qPCR in cultured cardiac nonmyocytes, identified by the presence of PECAM (CD31) mRNA and absence of α-MyHC mRNA (Online Figure V). The PECAM (CD31) marker indicated that the nonmyocytes included endothelial cells, the predominant nonmyocyte (>60% of nonmyocytes).15,16 These nonmyocytes expressed substantial β2 and β3 mRNAs, but very little β1 mRNA, which was present in myocytes, as expected, and radioligand binding confirmed a high level of β3-AR protein in β1/2-KO nonmyocytes (Online Figure V). We did not attempt to identify fibroblasts because they are a minor nonmyocyte population (<20%), and markers are uncertain.15 However, β2-ARs are present and coupled to proliferation in cultured cardiac fibroblasts.17–20

In summary, β1 mRNA is present in all myocytes, with a 30-fold range from lowest to highest, and higher levels in the myocyte subpopulation that also has high levels of the α1A. β2 and β3 mRNA and binding are mostly absent in myocytes, but are substantial in nonmyocytes.

The β1-AR Activates PLN in All Myocytes; the β2 and β3 Do Not Activate PLN

Radioligand binding indicated sizable levels of β1-AR protein in myocytes (Figure 6C), but β-AR antibodies are not specific.21 As a test for β1-AR protein and function in individual myocytes, we used the robust phosphorylation of PLN by ISO on the PKA site, serine 16, visualized by immunocytochemistry, as illustrated in Figure 7A, left. As with pERK, mean pPLN fluorescence per cell was quantified by digital microscopy in ≈115 cells per group, and a cell positive for activation was defined as pPLN fluorescence greater than the median plus range of the VEH control, shown by the dotted horizontal lines. Each dot is 1 cell. By ANOVA with Newman–Keuls post test, there were no significant differences among VEH, A6 NR, and A6 AKO, whereas A6 PIE and A6 NIE were both significantly different from all 3 other groups (P<0.001). The table summarizes the results with A6 in WT cells, with a response >40% defined as high (dotted boxes in B). Every tested cell responded to ISO (Figure 7). NIE indicates negative inotropic effect; NR, no response to A6; and PIE, positive inotropic effect.

The α1A functions in a subset of wild-type (WT) myocytes to activate contraction. Sarcomere length (SL) was measured in paced myocytes treated with Vehicle (VEH), A61603 (A6; 100 nmol/L), and the β1/2-adrenergic receptor agonist isoproterenol (ISO; 1 μmol/L). A, Raw tracings of SL vs time, with VEH, A6, and ISO added at arrows. B, Cell contraction was quantified from the difference between diastolic and systolic SL, and a response to A6 was defined as a change in contraction more than the median±range of the VEH control, shown by the dotted horizontal lines. Each dot is 1 cell. By ANOVA with Newman–Keuls post test, there were no significant differences among VEH, A6 NR, and A6 AKO, whereas A6 PIE and A6 NIE were both significantly different from all 3 other groups (P<0.001). The table summarizes the results with A6 in WT cells, with a response >40% defined as high (dotted boxes in B). Every tested cell responded to ISO (Figure 7). NIE indicates negative inotropic effect; NR, no response to A6; and PIE, positive inotropic effect.
of vehicle-treated cells. The experiment in Figure 7A, right, shows that ISO activated PLN in 97% of WT myocytes, versus in 0% of β1-KO cells. The ISO response was blocked by propranolol, indicating β-AR specificity. Gi inactivation with pertussis toxin (PTX) had a small effect to increase pPLN equally in both WT and β1-KO myocytes (Figure 7A, right). In replicate immunocytochemistry experiments with myocytes from 9 WT and 3 β1-KO hearts, ISO activated PLN in 97±1% of WT myocytes versus 5±3% of β1-KO cells.

We used immunoblot to test further whether the β2 or β3 could activate PLN, as reported for the β2-AR.22–25 Online Figure VI shows that S16-pPLN blots were clean, and revealed no increase in pPLN in β1-KO myocytes, above that seen with PTX alone. Online Figure VI also shows an ISO concentration–pPLN response curve, indicating in WT myocytes an EC50 0.8 nmol/L with a maximum 10-fold at 10 nmol/L, and negligible activation in β1-KO myocytes. Multiple experiments summarized in Figure 7B show pPLN activation via a β-AR in WT myocytes, and no activation by ISO in β1-KO myocytes, even with PTX or the phosphodiesterase 4 inhibitor rolipram. We tested PTX and rolipram because they are reported to enhance β2-mediated phosphorylation of PLN and contraction in β1-KO neonatal myocytes,23,24,26 and in adult rat myocytes.22

In summary, the β1-AR stimulates PLN phosphorylation at S16 in all myocytes, and the β2 and β3 do not increase pPLN.

The β1-AR Activates eNOS in Myocytes; the β3 Does Not Activate eNOS

Activation of eNOS is implicated in cardioprotection by the β3-AR.27 Online Figure VII shows that ISO activates eNOS in WT myocytes, but not in β1/2-KO myocytes, providing additional evidence that the β3 is not functional in myocytes.

The β1-AR Stimulates Contraction in All Myocytes; the β2 and β3 Do Not Stimulate Contraction

As a second end point for β-AR subtype function in individual myocytes, we measured the contractile response to ISO in individual WT, β2-KO, β1-KO, and β1/2-KO myocytes. Figure 8A shows raw tracings of sarcomere length over time, and Figure 8B summarizes the results. All of 40 WT myocytes and all of 28 β2-KO cells had a positive inotropic effect with ISO, versus none of 10 β1-KO myocytes, pretreated with PTX to enhance any contractile response through the β2-AR.22–24,26 ISO stimulated contraction in 2 of 20 β1/2-KO cells, but these responses were only ≈10% of the responses seen with ISO in WT myocytes (Figure 8A). The β3-AR did not mediate a contractile response to ISO in β1/2-KO intact myocardium (Online Figure VIII). As a control, some β1/2-KO cells were still able to respond to A61603 (Figures 8A; Online Figure VIII). Conversely, as shown in Figure 8C, WT cells that did not respond to A61603, had sarcomere shortening with ISO identical to myocytes that did contract with A61603.

In summary, the β1-AR causes a positive inotropic response in all myocytes, and the β2 and β3 do not activate contraction.

Summary

Figure 9A summarizes the excellent agreement among the individual myocyte assays, indicating that all myocytes have the β1 and α1B, ≈60% have the α1A, and only ≈5% each have the β2 and β3. An average ≈20% of myocytes have high levels of the α1A in all 4 assays. Figure 9B summarizes the average number of receptor molecules per myocyte from radioligand binding assays in populations of isolated cells, as in Figure 6 and Online Figure IX. Finally, Figure 9C suggests a model for myocyte AR phenotypes, showing that the results define 4 different populations of myocytes according to which ARs are present.

Discussion

These data are the first to examine ARs in individual ventricular myocytes. Contrary to current models,1,2 we find that the β1 and β2 are not the predominant myocyte ARs. Instead, the dominant myocyte ARs are the β1 and the α1B, which are present in all cells. Also, contrary to most thinking, the β2 and β3 are at low levels in myocytes, but are abundant in heart, the β3-AR surprisingly so, and are present in cardiac nonmyocytes. The α1A, rather than being uniformly expressed at low levels in all myocytes, as we had assumed, is present in a 60% myocyte subpopulation. Furthermore, for the α1A, there was an interesting further subpopulation of 20% cells with high...
α1A expression and function. These findings suggest a re-evaluation of cardiac adrenergic signaling models and experimental approaches, as discussed below.

Because valid AR antibodies are lacking, 8,21,28–30 we used other assays of expression (genetic reporters, mRNAs) and function (signaling, contraction) to identify the ARs in individual cells. Although each assay has limitations, the excellent agreement among the assays for each AR and for the high α1A supports the accuracy of the conclusions (Figure 9A).

However, we were technically unable to do 2 assays in the same cell, for example, α1A mRNA and contraction in the same myocyte. A potential concern with enzymatically isolated cells was receptor proteolysis explaining the low levels of β2 and β3. Several findings alleviate this concern, including the complimentary results with the respective mRNAs; preserved function in signaling and contraction of the α1A and β1 in enzymatically isolated cells; previous functional studies of isolated neonatal β-AR KO myocytes (discussed below); and past work concluding β2 proteolysis did not alter function. 31 We also validated in the intact heart without cell isolation that the α1A is expressed and signals in only a subset of myocytes (Figure 1; Online Figures II and III; Online Movie).

The main limitation of our study was that we studied the mouse only, with the exception that we showed the α1A in a subpopulation of NRVMs (Online Figure IV). Using adult KO myocytes was crucial to our approach, to validate the selectivity of A61603 for the α1A, and to test the role of β-AR subtypes. Many studies report β2-AR effects in cardiac myocytes of various species. 22,25,31–44 However, these conclusions are based largely on drug dosing with questionable selectivity. Specifically, the agonist zinterol, used to identify the β2, has 9 nmol/L affinity for the cloned β2-AR and 1 μmol/L affinity for the cloned β1-AR. 45 Thus, concentrations of zinterol over ≈80 nmol/L would lose selectivity for the β2, and most studies use higher dosing. 22,25,31,32,34–36,39,40,42–44 Indeed, we find that zinterol increases pPLN in β2-KO cells as effectively as does ISO, with an EC50 ≈200 nmol/L (Myagmar and Simpson, unpublished data, 2017). Similarly, the antagonist ICI-118551, also used to identify the β2-AR, usually with zinterol as agonist, has 3 nmol/L affinity for the cloned β2-AR and 500 nmol/L affinity for the cloned β1-AR. 46,47 Thus, concentrations of ICI-118551 over ≈30 nmol/L would lose selectivity for the β2-AR, and most studies use higher dosing. 32–34,37,41,44
ICI-118551 drug dosing could have resulted in overestimation of β2-AR effects in myocytes.

Similarly, the β3-AR has been identified in myocytes mainly by an antibody, which has uncertain specificity, and by the agonist BRL37344, which is not selective for the β3, or even for ARs. Few radioligand binding studies exist for the β2 and β3.

Competition radioligand binding for the β2-AR in rat adult myocytes is discordant; one study finds no β2 and another finds 17% β2. Given the difficulty of drug identification of the β-AR subtypes, there are surprisingly few studies using β-AR KO myocytes to study the receptors. One group reported 17% of total β-AR binding was still present in adult β1-KO cells, suggesting the β2 and/or β3 in myocytes. This study used single-point binding assays, which might explain the difference from our finding of no detectable binding in the β1-KO (Figure 6). Also surprising, we find no previous reports of β-AR binding in β1/2-KO hearts or myocytes, to quantify the β3. We find that the β3 is far more abundant in heart than is appreciated currently, comprising ≈20% of total β-ARs (Figure 6), most likely in endothelial cells (Online Figure V).

Another group has extensive evidence for β2 and β3 signaling and contraction effects in β-AR KO neonatal mouse myocytes. These results do not prove necessarily that the β2 and β3 are in a high percent of neonatal myocytes because the studies are done in uniformly beating cell syncytia, where a few cells can drive contraction, and β2 or β3 stimulation of contaminating nonmyocytes can secrete paracrine factors for myocytes. However, we did not study neonatal mouse myocytes.

Our data do not answer whether each cell has over its lifetime only one of the 4 AR phenotypes shown in Figure 9C. However, we do believe that these phenotypes are likely stable over time in the normal adult heart because we studied cells from mice aged ≈8 to 20 weeks. Furthermore, transcript variability in individual cells is deterministic, not stochastic, in which case the same cells would have the same receptors over their lifetimes.

Our data have important experimental and biological implications. Experimentally, a first caution is that β2 and β3 overexpression in myocytes that do not express them normally is a nonphysiological approach to identify cardioprotection or novel signaling. Second, β2, β3, and α1A electrophysiology and excitation-contraction coupling studies in isolated myocytes need to keep in mind that a cell with no or minimal response might not be a bad cell, but simply a cell with no or few receptors. Group data that omit poorly responding cells could be misleading, for example, group data for the α1A...
contraction data in Figure 4 would be misleading. In the literature, only 2 of 51 reports on α1-AR effects in isolated cells report individual cell data, with a positive control for nonresponsive cells (Online Table I). Third, it is risky to assume that assays on large ensembles of cells reflect effects in all of the cells. In myocytes, for example, α1-ARs in cultured myocytes induce β-MyHC. This activation is assumed to reflect most or all myocytes and is used widely to study hypertrophic transcription. On the contrary, recent data show that β-MyHC is induced in hypertrophy in only a minor subpopulation of small myocytes, indicating the risks of generalizing from cell ensembles. Overall, these new data place limits and increase the difficulty of experimental approaches.

Biologically, these data suggest new paradigms in cardiac adrenergic physiology. First, the β1 and α1B are the intrinsic regulators of catecholamine responses in all myocytes, not the β1 and α2 as thought currently. Furthermore, because the β1 and α1B are the only ARs that coexist in all myocytes, it is natural to wonder if they interact, either functionally or physically. Little is known about the role of the α1B, but the predominant negative inotropic effect seen in our experiments raises the interesting question whether α1B activation by NE or EPI could serve as a “brake” on β1-AR inotropy. Testing this idea is challenging because there are no agonists selective for the α1B, and nonselective agonists active at the α1B also stimulate β-ARs. However, a cardiac transgenic supports this hypothesis, where α1B overexpression inhibits β-AR-mediated inotropy and adenylyl cyclase activation. Because NE has ≈10-fold lower affinity for the α1B versus the β1, an α1B-β1 interaction could be especially relevant in heart failure when β1-ARs are downregulated and NE levels are elevated.

Second, the presence of the β2 and β3 mainly on nonmyocytes suggests a reinterpretation of the observation that these receptors, unlike β1 receptors, are not downregulated in heart failure. Thus, maintenance or increase of β2 and β3 levels in heart failure is likely because of nonmyocyte proliferation, not regulation different from the downregulated β1 in myocytes. Furthermore, paracrine signaling by nonmyocytes, not direct effects on myocytes, would also seem to explain any myocyte contractile and protective effects of the β2 and β3. In this regard, some studies using β2-KO models indicate that β2
activation can be toxic, not protective as generally thought.\textsuperscript{67,68} Increased $\beta_2$ effects in nonmyocytes, such as fibroblast proliferation, could explain maladaptive effects of $\beta_2$ activation.

Third, the $\alpha_{1A}$ stimulates inotropy and protection throughout the whole heart,\textsuperscript{69,70} and a key question for further study is the mechanism, when the $\alpha_{1A}$ is present in only 60\% of myocytes, and at high levels in only 20\% (Figure 9). The $\alpha_{1A}$ is absent in nonmyocytes,\textsuperscript{17,71} so a nonmyocyte-to-myocyte mechanism similar to $\beta_2$ or $\beta_3$ effects is not relevant. Presumably the $\alpha_{1A}$ uses some myocyte-to-myocyte mechanism(s), perhaps involving secreted growth factors, exosomes, or gap junctions.

Finally, it seems likely that the 4 AR cell phenotypes defined by this study (Figure 9C) will determine distinct downstream signaling and myocyte phenotypes, given the ubiquitous regulation of myocyte biology by NE and EPI. Distinct ventricular myocyte phenotypes, as we show here, are rarely identified. However, a recent interesting study suggests that the hypertrophic cardiomyopathy phenotype could be explained by marked cell-to-cell differences in expression of mutant and WT $\beta$-myosin among individual myocytes.\textsuperscript{72} Accordingly, it will be important to develop new methods to phenotype individual myocytes and relate phenotypes to receptor expression (Lopez et al, unpublished data, 2017).

In summary, the $\beta_1$ and $\alpha_{1B}$ are the dominant cardiac myocyte ARs, present in all cells, where the $\alpha_{1B}$ might serve as a “brake” on $\beta_1$ inotropic effects. The $\beta_2$ and $\beta_3$ are each in only $\sim$5\% of myocytes, but both are abundant in nonmyocytes, a distribution that enlightens several aspects of $\beta_2$ and $\beta_3$ biology. The $\alpha_{1A}$ is in a 60\% subpopulation, and present at high levels in only 20\% of myocytes. Cardioprotective and inotropic signaling by the $\alpha_{1A}$ would seem to involve myocyte-to-myocyte mechanisms. The results define myocytes with 4 distinct AR phenotypes, which are likely to determine distinct downstream myocyte phenotypes.

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We thank the Mouse Biology Program at the University of California, Davis, who constructed the $\alpha_{1B}$ reporter mouse.

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Disclosures
None.
References


Adrenergic Receptors in Individual Myocytes


Adrenergic Receptors in Individual Ventricular Myocytes: The Beta-1 and Alpha-1B Are in All Cells, the Alpha-1A Is in a Subpopulation, and the Beta-2 and Beta-3 Are Mostly Absent

Bat-Erdene Myagmar, James M. Flynn, Patrick M. Cowley, Philip M. Swigart, Megan D. Montgomery, Kevin Thai, Divya Nair, Rumita Gupta, David X. Deng, Chihiro Hosoda, Simon Melov, Anthony J. Baker and Paul C. Simpson

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http://circres.ahajournals.org/content/suppl/2017/03/10/CIRCRESAHA.117.310520.DC2
Title: Adrenergic Receptors in Individual Ventricular Myocytes: the Beta-1 and Alpha-1B Are in All Cells, the Alpha-1A Is in a Subpopulation, and the Beta-2 and Beta-3 Are Mostly Absent

Contents:
• Online Figures I-IX
• Online Table I
• Detailed Methods

• Movies (2 Online Movies are included, showing Optical Projection Tomography (OPT) of βGal-stained AKO and WT hearts.)
Online Figure I. Validation of bGal as an index of α1A transcription, and bGal from none to high in AKO knockin myocytes from heart regions.

A. Left, bGal as a valid marker of α1A mRNA

![Graph showing bGal ACO (RLU/heart) vs. α1A mRNA WT (AU/βActin)]

- r2=0.94, p=0.001
- w12-16 vs. w3-4
- d10 vs. d5
- d15 vs. d10

![Graph showing α1 mRNA (AU/Actin)]

- α1A mRNA was assayed by RT-qPCR, and bGal enzyme activity was quantified with a chemiluminescence assay. Each value is mean ± SE, n=3-7 hearts; r2 is by linear regression; dotted lines are 95% confidence limits.

B. bGal from none to high in AKO myocytes

- No bGal (0 dots)
- 1-3 dots
- 4-6 dots
- ≥ 7 dots

![Images showing bGal staining in AKO myocytes]

C. bGal-expressing myocytes in heart regions

<table>
<thead>
<tr>
<th>Hearts</th>
<th>No Dots (%) cells</th>
<th>Dots (%) cells</th>
<th>% Cells with # Dots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>1-3</td>
<td>4-6</td>
</tr>
<tr>
<td>RV &amp; LV</td>
<td>13</td>
<td>38 ± 1</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>RV FW</td>
<td>9</td>
<td>41 ± 4</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>Septum</td>
<td>9</td>
<td>36 ± 2</td>
<td>64 ± 2</td>
</tr>
<tr>
<td>LV FW</td>
<td>9</td>
<td>41 ± 3</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>Average</td>
<td>(%) myocytes</td>
<td>39</td>
<td>61</td>
</tr>
</tbody>
</table>

Online Figure I. Validation of bGal as an index of α1A transcription, and bGal from none to high in α1A-KO knockin myocytes from heart regions.

A. Left, bGal in AKO hearts and α1A mRNA in WT hearts were quantified at various days (d) or weeks (w) after birth. α1A mRNA was assayed by RT-qPCR, and bGal enzyme activity was quantified with a chemiluminescence assay. Each value is mean ± SE, n=3-7 hearts; r2 is by linear regression; dotted lines are 95% confidence limits. Right, Heart α1 mRNAs during development. B. Dots of bGal, a reporter for α1A transcription, vary from none to high in AKO adult myocytes stained for bGal; original 100X. C. Dots of bGal stain were counted by stereomicroscopy in at least 400 cells per heart or region from combined ventricles (RV & LV), or different regions (FW=free wall); dot numbers were grouped as indicated; mean ± SE. Among total ventricular myocytes ~60% were LVFW, ~20% septum, and ~20% RVFW.
Online Figure II. bGal dots are observed in α1A-KO tissue section.

Adult WT and AKO hearts were stained with Bluo-Gal by the Langendorff method, and 10 μm paraffin sections were counterstained with Nuclear Fast Red. Color images were made by Nikon E600 microscopy. Arrows point to dots of bGal reaction product in AKO myocytes.
Online Figure III. An α1A agonist activates PKCδ in a small subset of myocytes in the intact WT heart, PMA activates PKCδ in all cells. WT hearts were perfused by the Langendorff method 10 min at 37°C with the β-antagonist timolol 2 μmol/l, then with addition of Vehicle (100 μmol/l ascorbic acid in Krebs buffer), the α1A agonist A61603 100 nmol/l, or PMA 100 nmol/l. After 5 min, when PKC activation was maximum, 8 μm frozen sections were cut from ventricle and stained with anti-PKCδ (red), and bisbenzamide for nuclei (blue). Agonist doses and time were maximum, as assayed by translocation of PKCδ immunoreactivity from the cytosolic to the membrane fraction (data not shown).

**Top panels.** Arrows indicate examples of nuclei with positive PKCδ staining (red).

**Bottom panel.** Percent nuclei positive for PKCδ staining among 70-110 nuclei (mean 87) for the 3 conditions shown, plus negative control (NoAb, bis-benzamide with no 2° antibody, micrograph not shown); values are mean ± SE for counts by 3 independent, blinded observers.
Online Figure IV. The α1A functions in a subset of NRVMs to activate ERK. Cultured NRVMs were treated with agonist or vehicle for 5 min, at maximum ERK activation, then fixed and stained for total myosin (MF20 Ab, red) and pERK (green). A. The α1A agonist A61603 (200 nmol/l) activates ERK in 2 cells (left, “Pos” green fluorescence) of the 3 shown (right, myosin). Original 100x. B. pERK mean fluorescence units per myocyte (units/cell area) was quantified in 240-290 individual NRVMs after treatment with A61603 or PMA (100 nmol/l) as a positive control. Each dot is one cell. Percent values indicate the fraction of cells with fluorescence greater than the vehicle median plus range. C. The percent of myocytes positive for pERK fluorescence was counted in ≥200 NRVMs in each of 5 separate cultures; bars are mean±SE, with p by ANOVA and Newman-Keuls post-test. A61603 activates ERK in 67±2% of cells, vs. 7±3% in vehicle, and PMA activates ERK in 90±1%.
Online Figure V. The β2- and β3-ARs are expressed in ventricular nonmyocytes.

Ventricular myocytes (MCs) and nonmyocytes (NMCs) were isolated from the adult mouse heart. NMCs were cultured for 3 days in medium with 10% calf serum. RT-qPCR quantified mRNA levels.

A. Nonmyocyte (NMC) and myocyte (MC) mRNA markers

<table>
<thead>
<tr>
<th>mRNA Marker</th>
<th>NMC AU to HKGs</th>
<th>MC AU to HKGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PECAM1</td>
<td>20000</td>
<td>500</td>
</tr>
<tr>
<td>α-MyHC</td>
<td>75000</td>
<td>225000</td>
</tr>
</tbody>
</table>

B. β-AR mRNAs in WT NMCs and MCs

<table>
<thead>
<tr>
<th>β-AR mRNA</th>
<th>NMC AU to HKGs</th>
<th>MC AU to HKGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1-AR</td>
<td>150</td>
<td>500</td>
</tr>
<tr>
<td>β2-AR</td>
<td>225000</td>
<td>225000</td>
</tr>
<tr>
<td>β3-AR</td>
<td>30000</td>
<td>30000</td>
</tr>
</tbody>
</table>

C. β3-AR Binding in β1/2 KO NMCs and MCs

Online Figure V. The β2- and β3-ARs are expressed in ventricular nonmyocytes.

Ventricular myocytes (MCs) and nonmyocytes (NMCs) were isolated from the adult mouse heart. NMCs were cultured for 3 days in medium with 10% calf serum. RT-qPCR quantified mRNA levels.

A. NMCs are identified by PECAM (CD31) mRNA and no α-MyHC mRNA. PECAM mRNA in the MC preparations suggests NMC contamination.

B. β1 mRNA is in WT MCs, whereas β2 and β3 mRNAs are present in WT NMCs.

C. β3 binding is abundant in membranes from β1/2 KO NMCs. Estimated Bmax is at least 366 fmol/mg, although the exact value is not reliable, since binding does not saturate.
Online Figure VI. Immunoblot showing the β1-AR phosphorylates S16-PLN. Cultured WT or β1KO adult mouse myocytes were treated with ISO or vehicle for 5 min, when S16-phosphorylated PLN (pPLN) was maximum, and cells were harvested for pS16-PLN immunoblot. A. Loading control, the blot stained with coomassie blue. B. Complete immunoblot. C. pPLN bands showing groups; ISO (10 nM), L-propranolol (1 μM), dbcAMP (5 mM), or vehicle (ascorbic acid 100 μmol/l); some cells were pretreated for 3 h with pertussis toxin (PTX) 0.3 mg/ml. The 2nd lane from the right (X) was disrupted after loading. D Summary concentration-response relationships for ISO in WT myocytes and β1KO myocytes (n = 4 hearts WT, 3 β1KO).
A. ISO activates p-eNOS in WT not β1/2-KO myocytes

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>β1/2-KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISO/PTX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S1177-eNOS (140 kd)

B. p-eNOS in WT and β1/2-KO myocytes

Online Figure VII. The β3-AR does not activate eNOS in β1/2-KO myocytes. Cultured adult mouse myocytes were treated 15 min with vehicle, ISO (100 nmol/l), or ISO plus PTX (750 ng/ml) for 15 min, and examined for eNOS phosphorylated (activated) at S1177. A. Immunoblot shows increased p-eNOS in WT myocytes with ISO and ISO/PTX, but not in β1/2-KO myocytes. β1/2-KO myocytes have lower total eNOS and slight baseline activation. B. Left, Immunochemistry shows increased p-eNOS in a WT myocyte with ISO, but not in a β1/2-KO myocyte; inset is phase contrast of the cell examined. Original 100x. Right, p-eNOS mean fluorescence units per myocyte (units/cell area) was quantified in individual WT and β1/2-KO myocytes after treatment with ISO or vehicle. Each dot is one cell. The dotted line indicates vehicle+range. ISO does not increase p-eNOS in β1/2KO myocytes. WT ISO is greater than all other groups (p<0.001 by ANOVA).
Online Figure VIII. Isoproterenol (ISO) does not change contraction in an LV trabecula from β1/2-KO heart. Electrically stimulated force was measured in an LV trabecula paced at 0.5Hz and superfused at room temperature in oxygenated Krebs buffer (1.1 mmol/l calcium). ISO was added at arrows; A61603 (A6, 100 nmol/l) was tested after ISO, and caused a typical biphasic response.
A. α1-AR Binding in WT isolated myocytes

![Graph showing total α1-AR binding and 5MU competition binding with percentage maximum bound.](image)

B. α1-AR Binding in WT and KO hearts

![Graph showing saturation radioligand binding in WT and KO hearts.](image)

C. Summary of binding in isolated myocytes

<table>
<thead>
<tr>
<th></th>
<th>Measured Average ARs per Cell (a)</th>
<th>Measured α1-AR Subtypes (%) (b)</th>
<th>Calculated Average ARs per Cell (c = a x b)</th>
<th>Estimated Average ARs per Cell in 60% Expressing (c/0.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β1-ARs</td>
<td>55,199 ± 3,285 (11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-ARs</td>
<td>25,633 ± 2,605 (10)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>α1A</td>
<td>19 ± 1 (12)</td>
<td></td>
<td>4,870</td>
<td>8,117</td>
</tr>
<tr>
<td>α1B</td>
<td>81 ± 1 (12)</td>
<td></td>
<td>20,763</td>
<td></td>
</tr>
</tbody>
</table>

Online Figure IX. α1-AR radioligand binding.

A. Examples of left, saturation radioligand binding in isolated myocytes (n = 2 WT hearts); and right, competition binding with 5-methylurapidil, an α1A-selective antagonist, showing components with high (15%) and low (85%) affinity (n = 3 WT hearts). B. Saturation radioligand binding in WT and KO hearts (n = 3 hearts each genotype). The percent receptors detected in the α1B KO (i.e. α1A receptors) is similar to that identified by competition binding. C. Summary of β-AR and α1-AR binding (n = hearts). The average α1A-ARs per myocyte in the last column is calculated, since only 60% of the cells used for assay actually express the receptor.
### Online Table I. Studies of alpha-1-AR effects in isolated myocytes

<table>
<thead>
<tr>
<th>YEAR &amp; FIRST AUTHOR</th>
<th>SPECIES</th>
<th>CELLS</th>
<th>AGONIST</th>
<th>ALPHA-1 EFFECT</th>
<th>REF</th>
</tr>
</thead>
<tbody>
<tr>
<td>No individual cell data (group data only)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1988 Apkon</td>
<td>Rat</td>
<td>AVM</td>
<td>PE, methoxamine</td>
<td>Inhibit the calcium-independent voltage-activated outward potassium currents, prolong APD</td>
<td>1</td>
</tr>
<tr>
<td>1988 Hescheler</td>
<td>Rabbit &amp; Guinea pig</td>
<td>AVM</td>
<td>NE</td>
<td>In rabbit no effect on slow inward calcium current (but increase contractility in papillary muscle); in guinea pig no effect on calcium current or contractility</td>
<td>2</td>
</tr>
<tr>
<td>1989 Fedida</td>
<td>Rabbit</td>
<td>AAtM</td>
<td>NE, PE, methoxamine</td>
<td>Inhibit transient outward potassium current, prolong APD</td>
<td>3</td>
</tr>
<tr>
<td>1989 Ravens</td>
<td>Rat</td>
<td>AVM</td>
<td>NE</td>
<td>Inhibit outward potassium current</td>
<td>4</td>
</tr>
<tr>
<td>1990 Fedida</td>
<td>Rabbit</td>
<td>AAtM</td>
<td>NE, PE, methoxamine</td>
<td>Inhibit voltage-activated transient outward current, prolong APD, increase contractility</td>
<td>5</td>
</tr>
<tr>
<td>1990 Tohse</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit transient outward current (prolong APD, increase contractility in papillary muscle)</td>
<td>6</td>
</tr>
<tr>
<td>1991 Fedida</td>
<td>Rabbit</td>
<td>AVM</td>
<td>NE, methoxamine</td>
<td>Inhibit inward rectifier potassium current, IK1, in both the inward and outward directions</td>
<td>7</td>
</tr>
<tr>
<td>1991 Wang</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit (alpha-1A and alpha-1B required) voltage-activated transient outward current</td>
<td>8</td>
</tr>
<tr>
<td>1992 Terzic</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Increase or decrease contractility via pH changes and myofilament calcium sensitivity</td>
<td>9</td>
</tr>
<tr>
<td>1992 Boutjdir</td>
<td>Rat</td>
<td>AVM</td>
<td>NE</td>
<td>Inhibit beta-adrenergic-stimulated whole cell L-type calcium current</td>
<td>10</td>
</tr>
<tr>
<td>1992 Braun</td>
<td>Rabbit</td>
<td>AAtM</td>
<td>methoxamine</td>
<td>Inhibit the inwardly rectifying potassium background current (IK1) and the muscarinic cholinergic receptor-activated potassium current (IKACh)</td>
<td>11</td>
</tr>
<tr>
<td>1992 Tohse</td>
<td>Guinea pig</td>
<td>AVM</td>
<td>PE</td>
<td>Stimulate delayed rectifier potassium current (shorten APD)</td>
<td>12</td>
</tr>
<tr>
<td>1995 Strang</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit unloaded shortening velocity (measured in skinned cells)</td>
<td>13</td>
</tr>
<tr>
<td>1995 Duan</td>
<td>Rabbit</td>
<td>AAtM</td>
<td>PE, NE</td>
<td>Inhibit (alpha-1A) volume-sensitive chloride currents</td>
<td>14</td>
</tr>
<tr>
<td>1995 Iyadomi</td>
<td>Guinea pig</td>
<td>AVM</td>
<td>NE, PE</td>
<td>Inhibit the beta-adrenergic-activated chloride current</td>
<td>15</td>
</tr>
<tr>
<td>1995 Sato</td>
<td>Human</td>
<td>AAtM</td>
<td>methoxamine</td>
<td>Inhibit the inwardly-rectifying potassium channel (IK1)</td>
<td>16</td>
</tr>
<tr>
<td>1996 Chen</td>
<td>Rat</td>
<td>AVM</td>
<td>NE</td>
<td>Inhibit beta-adrenergic-stimulated unitary calcium currents</td>
<td>17</td>
</tr>
<tr>
<td>1996 Maki</td>
<td>Rat</td>
<td>NVM</td>
<td>PE</td>
<td>Reduce the number of functional calcium channels (chronic treatment)</td>
<td>18</td>
</tr>
</tbody>
</table>
### Supplementary Material: ARs in Individual Myocytes

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Tissue</th>
<th>Treatment</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996</td>
<td>Van Wagoner Rat</td>
<td>AAtM</td>
<td>PE</td>
<td>Inhibit outward potassium currents</td>
</tr>
<tr>
<td>1996</td>
<td>Oleska Guinea pig</td>
<td>AVM</td>
<td>NE, methoxamine</td>
<td>Inhibit the beta-adrenergic-activated chloride current</td>
</tr>
<tr>
<td>1996</td>
<td>Li Human</td>
<td>AAtM</td>
<td>PE</td>
<td>Inhibit the ultrarapid delayed rectifier potassium current; beta-adrenergic stimulation increases</td>
</tr>
<tr>
<td>1997</td>
<td>Hool Guinea pig</td>
<td>AVM</td>
<td>methoxamine</td>
<td>Inhibit the beta-adrenergic-activated chloride current, via pertussis toxin insensitive G protein</td>
</tr>
<tr>
<td>1998</td>
<td>Gaughan Rat</td>
<td>NVM</td>
<td>PE</td>
<td>Decrease L-type calcium and transient outward potassium current densities, prolong APD; without changing sodium or T-type calcium current densities (chronic treatment)</td>
</tr>
<tr>
<td>1999</td>
<td>Woo Guinea pig</td>
<td>AVM</td>
<td>PE</td>
<td>Concordant transient decrease then sustained increase in L-type calcium current, intracellular calcium transient, and contractility</td>
</tr>
<tr>
<td>1999</td>
<td>Yue Dog</td>
<td>AAtM</td>
<td>PE</td>
<td>Stimulate the ultra-rapid delayed rectifier potassium current, similar to beta-adrenergic stimulation, decrease APD</td>
</tr>
<tr>
<td>2000</td>
<td>Qu Rat</td>
<td>NVM &amp; AVM</td>
<td>NE</td>
<td>Inactivate (alpha-1B) pacemaker current (chronic treatment plus NPY)</td>
</tr>
<tr>
<td>2000</td>
<td>Satoh Rat</td>
<td>AVM</td>
<td>NE</td>
<td>Decrease SERCA and RYR mRNAs, calcium transient, and contractility, and increases NCX mRNA and protein (chronic treatment)</td>
</tr>
<tr>
<td>2001</td>
<td>Belevych Guinea pig</td>
<td>AVM</td>
<td>methoxamine</td>
<td>Inhibit beta-adrenergic-stimulated L-type calcium current, via tyrosine kinase</td>
</tr>
<tr>
<td>2001</td>
<td>Wang Dog</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit inward rectifier (alpha-1D) and transient outward (alpha-1A) potassium currents</td>
</tr>
<tr>
<td>2001</td>
<td>Cho Mouse</td>
<td>AAtM</td>
<td>PE</td>
<td>Inhibit the muscarinic cholinergic receptor-activated potassium current (IK Ach)</td>
</tr>
<tr>
<td>2003</td>
<td>Tateyama Mouse (LQT-3 mutant)</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit sodium channel bursting in disease-linked mutation</td>
</tr>
<tr>
<td>2003</td>
<td>Ross Mouse (WT &amp; activated alpha-1B TG)</td>
<td>AVM</td>
<td>PE</td>
<td>Over-expressed alpha-1B inhibits alpha-1A-mediated contractility</td>
</tr>
<tr>
<td>2004</td>
<td>Norby Mouse (WT &amp; IGF1 TG)</td>
<td>AVM</td>
<td>PE</td>
<td>Diabetes (STZ) and IGF1 over-expression do not alter alpha-1 negative inotropic effect</td>
</tr>
<tr>
<td>2004</td>
<td>Choisy Rat</td>
<td>AVM</td>
<td>PE, A61603</td>
<td>Inhibit (alpha-1A) an outwardly rectifying steady-state potassium current, distinct from previously characterized cardiac potassium channels</td>
</tr>
<tr>
<td>2004</td>
<td>Bian Rabbit</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit (alpha-1A) rapid delayed rectifier potassium current</td>
</tr>
</tbody>
</table>
## Supplementary Material: ARs in Individual Myocytes

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Model</th>
<th>Treatment</th>
<th>Effect</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>O-Uchi</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Increase L-type calcium current, through CaMKII; (IKr) HERG, via PIP2</td>
</tr>
<tr>
<td>2007</td>
<td>Yeh</td>
<td>Dog</td>
<td>AAtM</td>
<td>PE</td>
<td>Inhibit (alpha-1A) atrial hyperpolarization-activated, time-dependent constitutively active acetylcholine-regulated potassium current, via PKC</td>
</tr>
<tr>
<td>2007</td>
<td>Luo</td>
<td>Rat</td>
<td>NVM</td>
<td>PE, A61603</td>
<td>Increase (alpha-1A) calcium transients and sparks</td>
</tr>
<tr>
<td>2007</td>
<td>Alvarez</td>
<td>Mouse</td>
<td>AVM</td>
<td>PE</td>
<td>Increase pH</td>
</tr>
<tr>
<td>2008</td>
<td>O-Uchi</td>
<td>Rat</td>
<td>AVM</td>
<td>PE, A61603</td>
<td>Increase (alpha-1A) L-type calcium current, via Gq/11-PLC-PKC-CaMKII; decrease (alpha-1B) L-type calcium current via Go-beta-gamma</td>
</tr>
<tr>
<td>2008</td>
<td>Zitron</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit (alpha-1A) potassium current, via src tyrosine kinase and PKC</td>
</tr>
<tr>
<td>2009</td>
<td>Wang</td>
<td>Guinea pig</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit rapid component of delayed rectifier potassium current, via PKC and PKA</td>
</tr>
<tr>
<td>2010</td>
<td>Ichishima</td>
<td>Mouse</td>
<td>AVM</td>
<td>PE</td>
<td>Inhibit (alpha-1A) volume-regulated anion channels (chloride) and the regulatory volume decrease induced by hypotonicity, via Gq, PLC, and PIP2 depletion</td>
</tr>
<tr>
<td>2011</td>
<td>Mohl</td>
<td>Mouse (WT &amp; alpha-1A TG)</td>
<td>AVM A61603</td>
<td>Increase (alpha-1A) intracellular calcium, via snapin-TRPC6-stimulated calcium entry</td>
<td></td>
</tr>
<tr>
<td>2012</td>
<td>Jhun</td>
<td>Rat &amp; Mouse</td>
<td>NRVM, AMVM</td>
<td>PE</td>
<td>Stimulate voltage-gated L-type calcium channel (VLCC) expression at T-tubules and increase L-type calcium current density, via PKD and Rem1</td>
</tr>
<tr>
<td>2012</td>
<td>Bass</td>
<td>Rat</td>
<td>NVM</td>
<td>PE</td>
<td>Stimulate morphological hypertrophy phenotypes different from TNF-alpha, IGF-1, and fetal bovine serum (chronic treatment)</td>
</tr>
</tbody>
</table>

**Individual cell data, but without a positive control for non-responsive cells**

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Model</th>
<th>Treatment</th>
<th>Effect</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>Fedida</td>
<td>Rat</td>
<td>AVM</td>
<td>methoxamine</td>
<td>Increase contraction in 39% of cells, via inhibition of outward potassium current and prolonging APD</td>
</tr>
<tr>
<td>1998</td>
<td>Gambassi</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Increase (alpha-1A) or decrease (alpha-1B) contraction and pH</td>
</tr>
<tr>
<td>1998</td>
<td>Zhang</td>
<td>Rat</td>
<td>AVM</td>
<td>PE</td>
<td>Decrease then increase L-type calcium current in 67% of cells, depending on technique</td>
</tr>
</tbody>
</table>

**Individual cell data, with a positive control for non-responsive cells**
Supplementary Material: ARs in Individual Myocytes

<table>
<thead>
<tr>
<th>Year</th>
<th>Species</th>
<th>Location</th>
<th>AR Type</th>
<th>Effect 1</th>
<th>Effect 2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992</td>
<td>Gambassi</td>
<td>Rat AVM</td>
<td>PE</td>
<td>Increase calcium transient in 50% of cells; beta-AR stimulation increases in 100% of cells</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>1992</td>
<td>Failli</td>
<td>Guinea pig</td>
<td>AVM</td>
<td>Increase calcium transient in 33% of cells; potassium increases in 100% of cells</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

The table summarizes 51 papers from 1988 to 2012 that examined α1-adrenergic-mediated effects in isolated ventricular myocytes from various species. Studies are grouped according to whether they did or did not show individual cell data. The few papers that did show individual cell data, along with a positive control, agree with the present work that α1-AR effects are observed in myocyte subpopulations. AVM, adult ventricular myocyte; NVM, neonatal ventricular myocyte; AAtM, adult atrial myocyte. Myocytes are stated or assumed to be from male animals, except as noted.

Table References.

12. Tohse N, Nakaya H, Kanno M. Alpha 1-adrenoceptor stimulation enhances the delayed rectifier k+ current of guinea pig ventricular cells through the activation of protein kinase c. Circ. Res. 1992;71:1441-1446
Supplementary Material: ARs in Individual Myocytes


Supplementary Material: ARs in Individual Myocytes


DETAILED METHODS

ABBREVIATIONS.
3D, 3-dimensional
AKO, alpha-1A-adrenergic receptor knockout
AMVMs, Adult Mouse Ventricular Myocytes
bGal, beta-galactosidase
BDM, 2,3-butanedione monoxide
CBFHH, Calcium- and Bicarbonate-Free Hank’s salts with HEPES
Cq, quantification cycle
CYP, cyanopindolol
Ab, antibody
NMC, nonmyocyte
NRVM, neonatal rat ventricular myocyte
OPT, Optical Projection Tomography
PB, Perfusion Buffer: Na-HEPES-buffered calcium-free solution containing (mmol/l): NaCl 120.4, KCl 14.7, KH₂PO₄ 0.6, Na₂HPO₄ 0.6, MgSO₄·7H₂O 1.2, Na-HEPES 10, NaHCO₃ 4.6, taurine 30, 2,3-butanedione monoxide (BDM) 10, and glucose 5.5, all from Sigma Chemical (St. Louis, MO), except Na-HEPES, from Gibco BRL (Bethesda, MD).
PBS, phosphate buffered saline
pERK, phosphorylated ERK
RT-qPCR, reverse transcription quantitative real time polymerase chain reaction
pPLN, phosphorylated phospholamban
RLUs, relative light units
SB, Stop Buffer: PB with 12.5 μmol/l CaCl₂ and calf serum 10% to inactivate proteases (HyClone Defined Bovine Calf Serum SH30073)
TBS-T, Tris-buffered saline with Tween-20
WT, wild type

ANTIBODIES AND DRUGS.
pERK1/2, phospho-p44/42 MAPK (Thr202/Tyr204) (pERK) rabbit monoclonal Ab (D13.14.4E) Cell Signaling #4370, Beverly, MA.
pPLN S16, rabbit polyclonal Ab, Upstate #07-052, EMD Millipore, Bellerica, MA.
anti-total sarcomeric myosin heavy chain (MF20) mouse mAb, Clone MF-20, Developmental Studies Hybridoma Bank, Iowa.
PKCδ mouse monoclonal Ab, BD Transduction Laboratories #P36520-610397 (now #610397) goat anti-rabbit Alexa Fluor 488-conjugated secondary Ab, Molecular Probes #A11034, Eugene, OR.
anti-rabbit IgG, HRP-linked antibody, Cell Signaling #7074.
goat anti-mouse Cy3-conjugated secondary antibody #115-165-003, Jackson ImmunoResearch Lab, West Grove, PA.
normal goat IgG, Santa Cruz Biotechnology #sc-2028, Santa Cruz, CA

A61603, (A6) Tocris Bioscience #1052, Minneapolis, MN
L-ascorbic acid, Sigma-Aldrich #A7506, St. Louis, MO
phorbol 12-myristate 13-acetate (PMA), Sigma #P8139
β1/2 antagonist (S)-propranolol HCl, Fluca #82066
β2 antagonist ICI 118,551, Sigma #I127
β1/2-AR agonist L-isoproterenol HCl, Sigma #I6504
\(N^6, O^{2\prime}\)-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (dBcAMP), Sigma #D0260
Pertussis toxin (PTX), List Biological Labs #180, Campbell, CA
Rolipram, Tocris #0905

ANIMALS AND CELLS.

Mice and rats.
Animal care and use were approved by the IACUC at the San Francisco Veterans Affairs Medical Center and complied with Guide for the Care and the use of Laboratory Animals (NIH publication No.86-23, revised 1985).

Mice were primarily adult males age 8-20 weeks in the C57Bl/6J background. \(\alpha\)1A-KO reporter mice have bGal replacing exactly the first coding exon.\(^1\) The \(\alpha\)1B-KO mice had human placental alkaline phosphatase (hPLAP) replacing the first coding exon (see just below). \(\beta\)1/2-KO mice were from Jackson Labs (#003810) and in a mixed background (C57BL/6J, DBA/2, 129, FVB/N, CD-1); mice for study were obtained by backcross into C57Bl6J, then intercrossing littermates for KO and WT controls. In agreement with previous findings,\(^2\) the observed frequency of \(\beta\)1-KO appeared to be less compared with \(\beta\)2-KO and WT. Mice in PKC\(\delta\) experiments were in an FVBN/129 mixed background. Female Sprague-Dawley rats with newborn litters were from Charles River.

\(\alpha\)1B KO with human placental alkaline phosphatase (hPLAP) knockin
The Mouse Biology Program (MBP) at the University of California, Davis, constructed the mouse. The MBP made the Adra1b-PLAP KI vector using standard methods as follows. The mouse \(\alpha\)1B gene clone (BAC clone RP24-247D18) was from CHORI BAC PAC Resources Center (http://bacpac.chori.org/). The targeting vector contained a 5’ short arm of 2.2 kb and a 3’ long arm of 9.9 kb. Homologous recombination replaced exon 4 with the hPLAP gene and the neomycin-resistance gene driven by the phosphoglycerate kinase promoter. The hPLAP cassette contained a stop codon and an SV40 polyadenylation signal. The MBP electroporated the vector into C57BL/6N ES Cell line JM8.F6. This cell line is available from the KOMP Repository (www.komp.org). Resulting ES cell clones were screened for loss of the native allele and homologous recombination by long range PCR, and containing a single copy of the plasmid. Several clones passed these 3 screens. Clone Adra1b_3h3 was further screened and determined to be 71% euploid via simple chromosome spread and count. Cells of this clone were microinjected into BALB/cAnNHsd blastocysts, and transferred to e2.5 stage pseudopregnant recipients. Resulting chimera were screened for % ES Cell derived coat color, and those greater than 50% (7 males total) were mated to C57BL/6NTac females. Germline heterozygous mice were produced, and these were then mated to MMRRC strain C57BL/6-Tg(CAG-Flpo)1Afst/Mmucd, RRID: MMRRC:032247-UCD, for excision of the Neo selection cassette in the mutant mice. Neo excised mice were identified via PCR, and mated further to C57BL/6J mouse strain to remove the FLP transgene. Mice in this study were from filial (F) generations 4–5 in the C57BL/6J background. Wild-type (WT) littermates were controls. Routine genotyping was by PCR with a common \(\alpha\)1B 5’-flanking primer (CCAATCTCCTCCTGGCTATGG) and specific 3’ primers against exon 4 (GCCAGGTTTGACAATGAAGTAGTTGG) and hPLAP (CTGTCTGGCACATGTTGTCTACATTG).
Isolation of Adult Mouse Ventricular Myocytes (AMVMs).

AMVMs were isolated as described in detail by O’Connell et al. WT and KO mice had deep anesthesia with isoflurane 3% in oxygen 0.5-1L/min and intra-peritoneal injection of heparin 0.5 ml 100 IU/ml in PBS, to prevent coagulation of blood in the coronary arteries. The heart was removed by cutting the transverse aorta between the carotid arteries, and placed in a 60mm dish at room temperature containing 10 ml of Perfusion Buffer (PB): Na-HEPES-buffered calcium-free solution containing (mmol/l): NaCl 120.4, KCl 14.7, KH2PO4 0.6, Na2HPO4 0.6, MgSO4-7H2O 1.2, Na-HEPES 10, NaHCO3 4.6, taurine 30, 2,3-butanedione monoxime (BDM) 10, and glucose 5.5, all from Sigma Chemical (St. Louis, MO), except Na-HEPES, from Gibco BRL (Bethesda, MD). After thymus and lungs were trimmed off, the heart was cannulated quickly onto a Langendorff perfusion apparatus, and perfused 4 min at 3 ml/min to flush blood from the vasculature. The heart was then digested for 2 min at 3 ml/min with PB supplemented with collagenase II 2.4 mg/ml (Worthington Biochemical, Lakewood, N.J.) without additional calcium, then for 8 min at 3 ml/min with PB with collagenase with additional CaCl2 40 μmol/l. Fifty ml digestion solution was prepared fresh for each heart and added to the reservoir just prior to digestion, to prevent heat inactivation of the enzyme. The absolute amount of enzyme varied somewhat from lot-to-lot.

The digested heart was transferred to a 60mm dish containing 10 ml Stop Buffer (SB) (PB with 12.5 μmol/l CaCl2 and calf serum 10% to inactivate proteases (HyClone Defined Bovine Calf Serum SH30073)). After atria and large vessels were removed, ventricular tissue was disrupted and dissociated by mincing into ~10-15 small pieces using fine tip forceps, then triturating sequentially (~10-15 times each) through plastic pipettes with 3 mm, 2 mm and 1 mm openings. After a 200 μl aliquot was taken to count cells, the whole cell suspension was centrifuged for 2 min at 50xg (Eppendorf Centrifuge 5804), the supernatant was removed, and the pellet was used as the myocyte fraction. For myocyte counts, duplicate 10 μl aliquots were used in a hemocytometer (VWR Catalogue 15170-079). The number of rod-shaped and round myocytes on each side of the hemocytometer in the duplicate aliquots was used to calculate the total number of myocytes and the percent rod-shaped myocytes.

AMVM culture.

WT and KO AMVMs were isolated as described above. Calcium was gradually reintroduced step-wise as detailed by O’Connell et al., from ~80 μmol/l to 100 to 400 to 900 μmol/l, and finally at plating to 1.2 mmol/l. Myocytes were plated for immunoblot at a density of 50 rod-shaped myocytes per mm² on laminin-coated 35 mm dishes (~40,000 per dish) (~800 mm² per dish), or for immunofluorescence at a density of 64 rod-shaped myocytes per mm² on laminin-coated one-well chamber slides (~60,000 per well) (Lab-Tec, NY; surface area, ~940 mm² per well), in 2 ml per well in MEM with Hank’s Balanced Salt Solution, supplemented with calf serum 10%, BDM 10 mmol/l, and penicillin 100 U/ml. After 2 h myocyte attachment, medium was changed to serum free MEM with Hank’s Balanced Salt Solution, supplemented with bovine serum albumin 1mg/ml, BDM 10 mmol/l, and penicillin 100 U/ml. Experiments were done after 24 h at 37°C in humidified air with 2% CO2 to maintain pH 7.3.

Neonatal Rat Ventricular Myocyte (NRVM) culture.

The current protocol is modified slightly from the original. Ventricles from 1-2 litters 1 day-old Sprague-Dawley rats were minced with fine scissors into 1-2mm³ pieces and digested at room temperature in a 50 ml Falcon tube with trypsin 1.5 mg/ml (BD Difco, Franklin Lakes, NJ) and DNAse 0.02 mg/ml (Sigma, MO), in 10 ml Calcium- and Bicarbonate-Free Hank’s salts with HEPES (CBFHH) containing (mmol/l): NaCl 137, KCL 5.36, MgSO4-7H2O 0.81, dextrose
5.55, KH₂PO₄ 0.44, Na₂HPO₄·7H₂O 0.34, and Na-HEPES 20 at pH 7.4. Tissue pieces were disrupted using a stir bar at the slowest speed (~30 rpm), and a 10 ml wide-tip serological pipette. The first 2 supernatants were removed at 10 min intervals and discarded. The next 4 dissociations at 7 min intervals were combined into a 50 ml tube with 7 ml calf serum to inhibit enzymes (HyClone Defined Bovine Calf Serum SH30073). This process was repeated 3-4 times for a total 12-16 dissociation steps. Cells in 50 ml tubes were centrifuged gently, re-suspended in MEM with Hank's salts (UCSF Media Service, San Francisco, CA), supplemented with calf serum 5%, penicillin G 50U/ml (Sigma, MO), and vitamin B12 1.5 mmol/l (Sigma, MO), combined in a single tube, and centrifuged and re-suspended again in the same culture medium.

To minimize contaminating non-muscle cells, cells were pre-plated for 30-60 min, and the myocyte-enriched cell suspension from the pre-plates was removed and counted. The yield of viable myocytes (excluding trypan blue) was approximately 3.5-4 million per neonatal heart. Myocytes were diluted to 60,000 cells/ml in MEM with calf serum 5%, plus BrdU100 mmol/l (Sigma, MO, #B5002 ), to inhibit any residual non-muscle cells, plated onto 2-well chamber slides (Lab-Tec, NY; surface area, 420 mm²/well) with a volume of 1 ml/well, and cultured at 37°C in humidified air with 1% CO₂ to maintain pH 7.3.

The next day, cultures were given 1 ml of serum-free Hank's MEM supplemented with vitamin B12, penicillin, BrdU, human transferrin 10µg/ml (#T2252), and bovine insulin 10µg/ml (#I1882) (all supplements from Sigma), and cultured for 2 h until agonist treatment.

Isolation and culture of adult mouse ventricular nonmyocytes (NMCs).

Adult mouse ventricular nonmyocytes were isolated using the Langendorff heart digestion method, as with AMVM (See “Isolation of AMVMs”). After anesthesia, the heart was cannulated and perfused for 4 min with PB to flush blood from the vasculature and then digested for 10 min with collagenase with CaCl₂ 40 µmol/l. To recover digested vascular cells the collagenase flow-through was collected into SB, and chilled on ice.

After digestion the heart was disrupted, dissociated in SB, and triturated sequentially using plastic pipettes. The whole cell suspension combined with the flow-through was centrifuged for 2 min at 50xg. The supernatant was pipetted twice through a 40 µm size cell strainer (Fisher Cat. # 22363547), and centrifuged for 5 min at 800xg. The pellet was resuspended in minimal essential media (MEM with Hank’s Balanced Salt Solution), supplemented with calf serum 10%, and penicillin 100 U/ml, plated onto 60 mm dishes, and cultured for 3 days at 37°C in humidified air with 2% CO₂.

BETA GAL AND HUMAN PLAP ASSAYS.

bGal enzyme activity in whole heart.

Under anesthesia with isoflurane, hearts were removed from AKO and WT mice, flushed to remove blood in phosphate buffered saline (PBS), containing (mmol/l) KH₂PO₄ 1.5, Na₂HPO₄·7H₂O 8, KCl 2.7, NaCl 137, and snap frozen in liquid N₂. Hearts were homogenized using a Polytron (PT10-35 GT, Kinematica) in 0.5-1.5 ml hypotonic extraction buffer (HEPES 25 mmol/l pH 7.5, MgCl₂ 5 mmol/l, EGTA 1 mmol/l, and Roche Complete Mini protease inhibitor cocktail one tablet per 10 ml). Duplicate 30 µl aliquots with 5, 10, and 15 µg protein (Bradford, Bio-Rad Protein Assay, #500-0006), covering the linear range of the bGal detection system in our experiments, were assayed in 12x75mm luminometer cuvettes (BD Monolight #556862).
bGal activity was measured using the Galacto-Star Chemiluminescent reporter Gene Assay System for the Detection of β-Galactosidase (Tropix, ABS180RS). Samples (30 μl each) were mixed with 200 μl reaction buffer, containing Galacto-Star substrate, and incubated at room temperature for 1 h, protected from light. Chemiluminescent signal in each tube was measured for 5 sec with a luminometer (Monolight 2010, Pegasus Scientific). bGal enzyme activity as relative light units (RLUs) per mg was calculated from the linear regression of activity versus protein using Microsoft Excel. No endogenous bGal activity was detected in WT heart.

Whole mount bGal staining of mouse heart tissue.

We followed the protocols of Pereira et al and Rokosh et al. Mice had anesthesia with isoflurane and intra-peritoneal injection of heparin 0.5 ml 100 IU/ml in PBS. The heart was removed by cutting the transverse aorta between the carotid arteries, and placed in a 60mm dish containing 10 ml of Perfusion Buffer (PB): Na-HEPES-buffered calcium-free solution containing (mmol/l): NaCl 120.4, KCl 14.7, KH2PO4 0.6, Na2HPO4 0.6, MgSO4-7H2O 1.2, Na-HEPES 10, NaHCO3 4.6, taurine 30, 2,3-butanedione monoxime (BDM) 10, and glucose 5.5, all from Sigma Chemical (St. Louis, MO), except Na-HEPES, from Gibco BRL (Bethesda, MD). One l of PB was made fresh from powder at the time of use, dissolving reagents in 18.2 MΩ H2O, sterilizing by filtration, and adjusting pH to 7.0 with sterile HCl as needed.

After thymus and lungs were trimmed off, the heart was cannulated quickly onto a Langendorff perfusion apparatus, and perfused 4 min at 4 ml/min with PB at 37°C to flush blood from the vasculature. The heart was next arrested in diastole by perfusion for 3 min in ice-cold PBS with 60 mmol/l KCl, then fixed 10 min by perfusion and submersion at room temperature in glutaraldehyde 0.2% (v/v) (from 25% stock; EM grade), formaldehyde 1.5% (v/v) (from 37% stock), EGTA 5 mmol/l (from 100 mmol/l stock, pH 8.0), MgCl2 2 mmol/l (from 1 mol/l stock), and sodium phosphate 100 mmol/l. Fixative was prepared fresh on the day of use and adjusted to pH 7.2.

After fixation, the heart was washed for 40 min by perfusion and submersion at room temperature in bGal Wash Buffer: MgCl2 2 mmol/l, sodium deoxycholate 0.01% (w/v) (from 1% w/v stock), Nonidet P-40 0.02% (v/v) (from 10% stock), and sodium phosphate 100 mmol/l. Wash buffer was prepared fresh on the day of use and adjusted to pH 7.2.

After washing, the heart was stained for 40 min by perfusion and submersion at room temperature in bGal Staining Solution with Bluo-Gal: bGal wash buffer containing Bluo-Gal 1mg/ml, K4Fe(CN)6 5 mmol/l, and K3Fe(CN)6 5 mmol/l. Bluo-Gal (halogenated-indolyl-b-D-galactoside) (Invitrogen, USA) was made fresh on the day of use as 25 mg/ml in dimethylsulfoxide, and was used for whole mount bGal staining because it is not soluble in organic solvents. The potassium ferrocyanide and ferricyanide stocks (Sigma, USA) were 100 mmol/l in sodium phosphate 100 mmol/l at pH 7.2, and were stored up to 1 y in the dark at 4°C. The staining solution was protected from light, and collected and reused during staining. After perfusion, the heart was stained an additional 24 h at 37°C with gentle rocking in a vial with Bluo-Gal solution (20:1 v/v reagent-to-tissue). After staining, the heart was washed three times for 30 min each in PBS at room temperature, and post-fixed by soaking in 3.7% formaldehyde at 4°C for 1 h to overnight.

The heart was then used either for Optical Projection Tomography (OPT) or paraffin sectioning.

Optical Projection Tomography (OPT) and 3-dimensional (3D) movie rendering.

Whole mount bGal stained hearts were washed repeatedly in distilled water and embedded in 1% low melting temperature agarose. Agarose blocks were affixed to metal OPT mounts, and trimmed to remove excess agarose. Tissue was dehydrated in multiple changes of
methanol over 2 d, then cleared by placing in benzyl alcohol/benzyl benzoate (2:1, v/v) in a polypropylene tube or glass vial (polystyrene dissolves in clearing agent) with gentle rocking for a minimum of 24 h.

Images were captured using Bioptomics OPT Scanner 3001M (Bioptomics, Edinburgh, UK) as described by Sharp et al.\textsuperscript{4}. Images were recorded through a 360° rotation of the specimen with a 0.9° step size, as viewed through a built-in microscope with a plan 4.0x-6.3x objective. Visible illumination (bGal signal) was from a 20W halogen lamp, and an autofluorescence view (surface signal) was captured with the GFP1 filter set (425/60 nm excitation, 480 nm emission) in the illumination and detection light path, to be able to reconstruct gross heart morphology. Illumination exposure time was equal in all WT and AKO hearts. The raw data (400 projected images) from each of the two channels were reconstructed into a pair of 3D voxel data sets, using in-house reconstruction software. 3D OPT reconstructions were then loaded into Bioptomics Viewer software for visualization. Volume renderings of the autofluorescence reconstruction (surface signal) were created using a green color map; and for bGal signal, a blue color map (dense staining was blue, lighter staining was grey). AKO bGal and surface volume renderings were exactly the same as for WT hearts. The 3D movie was generated using Quick Time Pro (Apple, CA).

**Paraffin sectioning.**

Whole mount bGal stained WT and AKO hearts after post-fixation in 3.7% formaldehyde were dehydrated through a graded ascending ethanol series, embedded in paraffin blocks, and sectioned at 10 μm. Dewaxed and re-hydrated tissue sections were counterstained with Nuclear Fast Red to identify cell morphology. Slides were observed by a light microscopy (E600, Eclipse; Nikon, Japan) using a 100x oil objective, and color images were made using a CCD digital camera (Spot PCI-CE Color; Model 1.3.0, Diagnostic Instruments, MI).

**Whole mount hPLAP staining of mouse heart tissue.**

We followed the protocols of Lobe and Guo.\textsuperscript{5} Mice had anesthesia with isoflurane and intra-peritoneal injection of heparin 0.5 ml 100 IU/ml in PBS. The heart was removed, cannulated onto a Langendorff apparatus, and perfused with PB (nominal calcium-free Na-HEPES buffer, see above "Whole mount bGal staining...") for 3 min. The heart was arrested in diastole by perfusion for 3 min with ice-cold PBS with KCl 60 mmol/l, then fixed 10 min by perfusion and submersion at room temperature in neutral buffered formalin (final 10%, Fisher #23-245-684).

After fixation, the heart was transferred into a preheated water bath, incubated 30 min in PBS at 70°C to inactivate endogenous alkaline phosphatase (AP) and then transferred back to the perfusion apparatus. The heart was perfused at room temperature for 5 min with PBS and for 10 min with alkaline phosphatase (alk phos) wash buffer (mmol/l): Tris-HCl 100 pH 9.5, NaCl 100, MgCl\textsubscript{2} 10.

After washing, the heart was stained for 40 min by perfusion and submersion at room temperature in alk phos staining solution with X-phos/NBT: AP wash buffer containing 5-bromo-4-chloro-3-indolyl phosphate (X-phos, Sigma-Aldrich, St. Louis, MO) 175 μg/ml, nitroblue tetrazolium (NBT, Sigma-Aldrich) 337 μg/ml, Levamisole 0.5 mmol/l, MgCl\textsubscript{2} 50 mmol/l, sodium deoxycholate 0.01% (w/v) (from 1% w/v stock), and Nonidet P-40 0.02% (v/v) (from 2% stock). The staining solution was prepared fresh on the day of use, adjusted to pH 9.5, and protected from light. After perfusion, the heart was stained an additional 24 h at room temperature with gentle rocking in a vial with X-phos/NBT solution (20:1 v/v reagent-to-tissue). After staining, the heart was washed three times for 10 min each in 0.1% Tween 20/ MgCl\textsubscript{2} 2 mmol/l in PBS. The heart was examined under a stereomicroscope (Zeiss SV11) and color images were made.
using a CCD digital camera (Spot PCI-CE Color; Model 1.3.0, Diagnostic Instruments MI).

**bGal staining and counting of isolated AMVMs**

AMVMs were isolated as just described, except in some experiments the RVFW, Septum, and LVFW were dissected out after digestion, to isolate myocytes specifically from those regions. Isolated myocytes were fixed with 0.05% glutaraldehyde in PBS for 10 min at 4°C, washed in PBS for 10 min and suspended in **bGal Staining Solution with X-Gal**: bGal wash buffer (see above) containing X-Gal 1mg/ml, K₄Fe(CN)₆ 5 mmol/l, and K₃Fe(CN)₆ 5 mmol/l. X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (Sigma, USA) was made fresh on the day of use as 25 mg/ml in dimethylsulfoxide. The potassium ferrocyanide and ferricyanide stocks were 100 mmol/l in sodium phosphate 100 mmol/l at pH 7.2, and were stored up to 1y in the dark at 4°C. The staining solution was protected from light.

After bGal staining for 24 h at 37°C, myocytes were counterstained with Nuclear Fast Red. Rod-shaped myocytes with dots of blue bGal reaction product were counted by light microscopy (E600, Eclipse; Nikon, Japan) using a 20x objective. Images were made using a CCD digital camera (Spot PCI-CE Color, Model # 1.3.0; Diagnostic Instruments, MI). No endogenous bGal staining was detected in WT myocytes. Round myocytes were also counted for total bGal positive cells, and % positive was the same as with rod-shaped (data not shown). However, dot counts were not reliable in rounded myocytes.

**Alk Phos staining of isolated AMVMs.**

We followed the protocols of Lobe and Guo. AMVMs were isolated as just described, fixed in neutral buffered formalin (final 10%) for 10 min at 4°C, washed in PBS, and then incubated 30 min at 70°C to inactivate endogenous alk phos. Cells were rinsed in **alk phos wash buffer** for 10 min and then stained in **alk phos staining solution with X-phos/NBT** (see above) at room temperature for 24 h.

After staining, the myocytes were washed in 0.1% Tween 20/ MgCl₂ 2mmol/l in PBS and examined under light microscopy (E600, Eclipse; Nikon, Japan). Images were made using a CCD digital camera (Spot PCI-CE Color, Model # 1.3.0, Diagnostic Instruments, MI). No endogenous AP staining was detected in WT myocytes.

**RNA ISOLATION AND ASSAYS.**

**RNA isolation from hearts.**

Under anesthesia with isoflurane, hearts were removed from AKO and WT mice, flushed in PBS to remove blood, and snap frozen in liquid N₂. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), following the “RNeasy Mini Protocol for Isolation of Total RNA from Heart, Muscle, and Skin Tissue”. Frozen heart tissue was suspended in a guanidine-isothiocyanate-containing lysis buffer and disrupted by pipetting (neonatal hearts), or was ground in a glass homogenizer (adult hearts), then disrupted using a QIAshredder spin column (Qiagen). The lysate was diluted and treated with proteinase K to remove contractile proteins that could interfere with RNA isolation, and debris was pelleted and discarded. Ethanol was added to the cleared lysate, and RNA was bound to the RNeasy silica-gel membrane. After multiple washings, total RNA was eluted in RNase-free water, and traces of DNA were removed with a Turbo DNAfree kit (Ambion, Austin, Texas). Samples with \( A_{260}/A_{280} \) ratio <1.9 (SmartSpec3000, Bio-Rad) were excluded from analysis.
Quantitative real time reverse transcription polymerase chain reaction (RT-qPCR) on RNA from heart.

One μg heart RNA isolated as above was reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen), random hexamers (Invitrogen), and oligo-dT (Roche, Nutley, New Jersey). qPCR was done in triplicate in an ABI PRISM 7900HT Sequence Detection System with 5% of the reverse transcription product, specific primers (125 nmol/l) that span the large intron in all α1-AR genes, and SYBR Green Master Mix (Roche) with ROX reference dye. Primers were as follows (5' to 3'):

- α1A forward, ATTGTGGTGGGATGCTTCGTCCT
- α1A reverse, TGGTTCCGGTGCTTGAAATTCGG
- α1B forward, CCTTGGGCATTGTAGTCGGAATGT
- α1B reverse, TTGAAGTAGCCCAGGACAACCT
- α1D forward, CTCTCGTAAGGCTGCTCAAGTTT
- α1D reverse, TGAAGACGCCCTGTGATGGTTTCA
- β-actin forward, TCATCACTATTGGCAACGAGCGGT
- β-actin reverse, TGCCACAGGATTCCATACCCAAGA

Data were analyzed with SDS software version 2.3 (Applied Biosystems, Foster City, CA). Relative quantization of PCR products used the ΔΔCq method, where arbitrary units were $2^{-\Delta\Delta Cq} \times 100,000$, Cq=quantification cycle, and $\Delta\Delta Cq=(\text{mean target gene Cq})-(\text{mean Cq of reference gene, β-actin})$.

RT-qPCR in single isolated AMVMs.

We measured β1 and α1A mRNAs in single AMVMs. Single cell isolation and capture. AMVMs were isolated from four WT male C57Bl/6J mouse hearts aged 12-15 weeks using the Langendorff heart digestion method, as described above ("Isolation of AMVMs"). As negative controls AKO and β1-KO AMVMs were isolated as well. After anesthesia, the heart was cannulated and perfused for 4 min with PB to flush blood from the vasculature and then digested for 10 min with collagenase. After digestion the heart was disrupted, dissociated in SB and triturated sequentially using plastic pipettes and centrifuged for 2 min at 50xg. Myocytes were re-suspended in PB, plated into one-well chamber slides (~20,000 per well) (Lab-Tec, NY), and examined in a dissection microscope. A borosilicate glass capillary pipette with ~ 50 μm tip diameter was placed in close proximity to a rod shaped myocyte, then the cell was drawn in with negative pressure. The cell and fluid (~ 5 μl) were then ejected into a PCR tube and flash frozen in liquid nitrogen.

Reverse transcription (RT). The cDNA synthesis reaction was done in 20 μl total volume. First, to ensure denaturation of RNA secondary structures, a mix of 5 μM oligo-dT (Roche, Nutley, New Jersey), 60 μM random hexamers (Roche, Nutley, New Jersey), and RT buffer were added directly to the ~ 5 μl frozen cell/PB and incubated at 65 °C for 10 min in a total volume 16 μl. The samples were cooled down on ice, and 1 mM dNTP, 20 U RNase inhibitor and 35 U Transcriptor Reverse Transcriptase (Roche, Nutley, New Jersey) were added. RT was done using the following temperature program: 25 °C for 10 min, 55 °C for 30 min, and 85 °C for 5 min.

Pre-amplification. The following reagents were mixed for 80 μl pre-ampl reactions: 12 μl primer pool, 40 μl SYBR Green Master Mix (Roche), 8 μl water, and 20 μl cDNA. The primer pool was made in water, 0.8 μM of each specific primer for α1A-AR, β1-AR, β-actin, and β2-
microglobulin (B2M). Primers were all intron spanning except beta1 as follows (5’ to 3’):

- α1A forward, ATTGTGGTGAGATGCTTGCTCC
- α1A reverse, TGTTTCCGGTGGCCTGAAATTC
- β1 forward, AGTGCTGCTTCGATCAGAACA
- β1 reverse, GCTCGCAGCTGTCGATCTTCT
- β-actin forward, TGACAGGATGCAGAAGGA
- β-actin reverse, CGCTCAGGAGGAGCAATG
- B2M forward, CTGACCGGCCTGTATGCTT
- B2M reverse, AGGCGGGTGGAACTGTGA

Pre-amplification PCR was performed on thermal cycler TC-512 (Techne, NJ) with the following conditions: an initiation of 95 °C for 3 min followed by 18 cycles of 95 °C for 20 sec, 57 °C for 4 min, 72 °C for 20 sec, and final elongation of 72 °C for 5 min. The resulting PCR product was diluted five times in water and used for the final qPCR.

qPCR. For the final qPCR, the following reagents were mixed for a 20 μl reaction volume: 2 μl 1.25 μM forward and reverse primer mix, 10 μl SYBR Green Master Mix (Roche), and 8 μl diluted pre-amped cDNA. Samples were run on an ABI PRISM 7900HT Sequence Detection System. The cycling program was 95 °C for 10 min as initiation, followed by 40 cycles of denaturation at 95 °C for 15 sec, and annealing at 60 °C for 1 min. After PCR, melting curves (Tm) were collected between 60 °C and 95 °C with 0.5 °C increments.

Data analysis. Data were analyzed with SDS software version 2.3.4 (Applied Biosystems, Foster City, CA). Three types of negative controls were done: KO mouse cells, no cell controls (chamber buffer), and no RT controls (genomic DNA). No quantification cycle (Cq) values were detected from any negative control. All products with undetected Cq values were replaced with 40, and Cq values with products giving rise to a double peak in Tm were removed. Products with undetectable Cq for reference genes (β-actin or B2M) were also removed. The data for α1A and β1 were normalized to β-actin and B2M as arbitrary units (AU) using the ΔΔCq method. AU values above the highest AU values in 2 AKO or 2 β1KO myocytes were defined as "expressed", whereas AU values at or below those seen in KO cells were defined as "not expressed".

Microarrays for mRNAs in single isolated AMVMs.

We quantified α1A, α1B, β2, β3, and MyHC mRNAs in single isolated myocytes using microarrays. AMVMs were isolated from 5 C57Bl/6J mouse hearts aged 20 weeks, 3 male and 2 female. Ten single cells from each heart were isolated under a microscope using a micropipette, and flash frozen in individual PCR tubes containing 1-2 μl of medium. Expression profiling used Sigma's WTA2 kit (Cat# WTA2) to amplify double stranded cDNA, as in Flynn et al.7 We used 2 rounds of amplification, since individual myocytes had low amounts of RNA. Single cells were extracted via the first step of Sigma's WTA2 protocol according to manufacturer's instructions. During this first step, the incubation with library synthesis buffer at 37°C for 5 min disrupted the membrane of the single cells and extracted the message for amplification. The library was prepared per the kit WTA2 kit protocol, and 1/5 of the library sample was added to 70 μl of the WTA2 amplification master mix, and cycled for amplification. After the first round of amplification, samples were purified using Qiagen’s QIAquick PCR Purification Kit (Cat# 28104) and processed on Qiagen’s Qiacube using the "Cleanup QIAquick PCR Amplification Reactions Standard V4" protocol; concentrated to a volume of 5 μl via speedvac; amplified a second time for 17 cycles (WTA2 amplification protocol and cycling parameters repeated); and purified using the QIAquick PCR purification kit. Product quality and
abundance were measured (Agilent Bioanalyzer), and 2 μg of cDNA was labeled with NimbleGen's One Color Labeling Kit (Cat# 0522355001).

The Cy3 labeled sample from a single cell was then hybridized to a 12x135K NimbleGen Gene Expression Array (Cat# 05543797001) according to the manufacturer's protocol. After hybridization, the slides were washed and dried, then scanned on a Molecular Devices GenePix 4200A Scanner with settings of 100POW and 300-350PMT. Pair files for each array were generated using NimbleGen's NimbleScan software and subsequently analyzed for each single cell. Each slide had 5000 random probes without homology to the mus musculus genome, used to calculate background for non-specific hybridization. These probes effectively estimated a level of "zero expression", as there are no transcripts with significant homology to these probes. Normalization for each array was carried out via the limma method, and the normalized values for each of the probes specific for Adra1a or other gene of interest were averaged. In most cases, there were at least 3 probes for each isof orm of each gene. There were two isoforms of Adra1a on the Nimblegen chip, AK085653 and BC113139. Males and females did not differ for the genes analyzed, and data were combined.

RT-qPCR on populations of isolated nonmyocytes and myocytes.

We measured β-ARs and cell markers (PECAM, α-MyHC) in populations of isolated nonmyocytes and myocytes. Isolated myocytes were lysed in Trizol reagent (Thermo-Fisher) and homogenized, and RNA was extracted using the RNeasy mini kit (QIAGEN). Isolated nonmyocytes after culture for 3 days were rinsed with PBS to remove dead cells, and total RNA was extracted using the RNAqueous-Micro Kit (Ambion), following the "RNAqueous-Micro Protocol" for micro-scale samples. Nonmyocytes were lysed and scraped off the dish using a guanidine isothiocyanate-containing lysis buffer included in the kit, homogenized by pipetting up and down, mixed with ethanol, and applied to a silica-based filter that selectively binds RNA. After multiple washings, total RNA was eluted in a very small volume of only 10 μl that allowed for recovery of a more concentrated RNA solution. The trace amounts of genomic DNA in both myocytes and nonmyocytes were removed by treatment with DNase. RNA quality and concentration was determined using an Agilent Bioanalyser.

For qPCR, one μg of total RNA was reverse transcribed with Transcriptor Reverse Transcriptase (Roche), random hexamers (Roche), and oligo-dT (Roche, Nutley, New Jersey). qPCR was done in triplicate in an ABI PRISM 7900HT Sequence Detection System with 5% of the reverse transcription product, specific primers (125 nmol/l), and SYBR Green Master Mix (Roche) with ROX reference dye. Primers were as follows (5’ to 3’):

β1 forward, AGTGCTGCGATTTGTCACAACA
β1 reverse, GCTCGCAGCTGATCCTTTTTA
β2 forward, TGCTATCACATGCCTTTC
β2 reverse, ACCACTCGGCTATTCTT
β3 forward, CAGCCAGCCTGTGGAAG
β3 reverse, CCTTCATAGCCATCAAACCTG
PECAM1 forward, CGGTGTTACGCGAGATCC
PECAM1 reverse, ACTCGACAGGGATGGAATCAC
MyHC (alpha) forward, CCAAGACTGTCCGAATGA
MyHC (alpha) reverse, TCCAAAGTGATCCTGATGA
β-actin forward, TCAATCTTGGCAACGAGCG
β-actin reverse, TGCACAGGTCATCCACGAAG
GAPDH forward, CTTTGGACTTGGTGAGGGC
GAPDH reverse, ACGGCCACGCTTTCCAGAG
Data were analyzed with SDS software version 2.3.4 (Applied Biosystems, Foster City, CA). Relative quantization of PCR products used the ΔΔCq method, where arbitrary units were $2^{-\Delta\Delta Cq} \times 100,000$, Cq=quantification cycle, and $\Delta\Delta Cq=(\text{mean target gene Cq})-(\text{mean Cq of reference gene, } \beta$-actin and GAPDH).

**ERK, PHOSPHOLAMBAN, AND PKCδ ASSAYS.**

**Phosphorylated ERK (pERK) immunofluorescence in cultured AMVMs.**

Cultured AMVMs from WT and AKO hearts after 24 h culture in serum free medium were treated 5 min with the $\alpha$1A-AR specific agonist A61603 (A6, 200 nmol/l), phorbol 12-myristate 13-acetate (PMA, 100 nmol/l), or vehicle control, L-ascorbic acid 100 μmol/l. Medium was aspirated; cells were fixed immediately in methanol/acetone (70:30, v/v) at -20°C for 20 min; washed with Tris-buffered saline with Tween-20 (TBS-T) 0.1% (v/v); and blocked with normal goat serum 5% (Jackson ImmunoResearch Lab, West Grove, PA) in TBS-T for 1 h at room temperature.

Fixed, washed, and blocked myocytes were then incubated overnight at 4°C with rabbit mAb to phospho-p44/42 MAPK (Thr202/Tyr204, Cell Signaling #4370), using a 1:50 dilution in TBS-T containing BSA 5%. The next day, cells were washed with TBS-T; incubated at room temperature for 1 h with goat anti-rabbit Alexa Fluor 488-conjugated secondary Ab, using a 1:400 dilution in TBS-T containing BSA 5%; washed with Triton X-100 0.2% in PBS, then with PBS; and finally mounted with Fluoromount G (Southern Biotech, AL).

Myocytes were examined by epifluorescence microscopy (E600, Eclipse; Nikon, Japan), equipped with a fluorescent filter cube (excitation 465 to 495 nm; dichroic mirror 505 nm; emission 515 to 555 nm, EF-4 FITC HYQ, Nikon) using 40x objective in 40-50 random fields. Fluorescent images of pERK in 40-50 random fields were taken with a CCD digital camera (Spot Insight 2Meg FW Mono; Model # 18.0, Diagnostic Instruments, MI) and Spot Advanced software (Version 4.5). Capture time was adjusted to 1 s for all sample groups. The intensity of pERK fluorescence was analyzed in at least 100 rod-shaped myocytes in each group in each experiment, using Quantity One software (Version 4.6.5; Bio-Rad, CA). The average number of myocytes studied was 219 ± 33 for 18 separate analyses.

In all experiments, the negative threshold was determined by examining fluorescence of the cells stained with nonspecific goat IgG as an isotype control antibody (Santa Cruz Biotechnology #sc-2028).

Fluorescent volume density (the total intensity of all the pixels in the cell per mm$^2$) of each myocyte was determined using the volume contour tool of the software, then raw values were exported to an Excel spreadsheet. Intensity values above vehicle-treated median + range fluorescence were considered positive. In every case, operators were blinded to genotype and treatment group.

Fluorescent and phase contrast images in the figures were made by epifluorescence microscopy with a 100x oil immersion objective using a CCD digital camera, and pseudo-colored using Spot Advanced software (pERK, green).

**Phosphorylated phospholamban (pPLN) western blot and immunofluorescence in cultured AMVMs.**

$\beta$-AR KO and WT AMVMs were cultured 24 h in serum free medium, pretreated 10 min with the $\beta$1/2-AR antagonist (S)-propranolol HCl (2 μmol/l), ICI 118,551 (50 nmol/l), rolipram (1
μmol/l), pertussis toxin (PTX, 0.3 μg/ml for 3 h at 37°C), or vehicle control (L-ascorbic acid, 100 μmol/l), then treated 5 min with the β1/2-AR agonist L-isoproterenol HCl (10 nmol/l-1 μmol/lSigma #I6504), or with N6,O2'-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (dbcAMP, 5 mmol/l). Preliminary experiments determined that 5 min was maximum pPLN.

For western blot, medium was aspirated, and cells were lysed in 100 μl 1X SDS sample buffer containing Complete Mini protease and phosphatase inhibitor cocktails 2 and 3. Lysates were stored at -80°C until immunoblot. Samples in SDS sample buffer were boiled for 5 min at 100°C, and equal numbers of cells (6,000 AMVMs per lane) were separated on Criterion Tris-HCl SDS-PAGE 18% gels. Proteins were transferred to membranes of PDVF, blocked with non-fat milk 5% in TBS-T, then incubated overnight at 4°C with primary antibody in BSA-Tween 5% (pPLN 1:10,000, Upstate #07-052). After wash membranes were incubated for 1 h at RT with secondary antibody in milk 5% in TBS-T (anti-rabbit IgG, HRP-linked antibody, Cell Signaling #7074).

Bands were developed using SuperSignal West Dura ECL reagent, images were captured with the ChemiDoc XRS system and analyzed with QuantityOne software version 4.6.9. Coomassie blue staining of the quantified blots confirmed equal loading of 6,000 AMVMs in each lane.

For immunofluorescence, medium was aspirated; and cells were fixed immediately in neutral buffered formalin (final 10%, Fisher #23-245-684), washed with Tris-buffered saline with Tween-20 (TBS-T) 0.1% (v/v), and blocked with normal goat serum 5% (Jackson ImmunoResearch Lab, West Grove, PA) in TBS-T for 1 h at room temperature.

Fixed, washed, and blocked myocytes were then incubated overnight at 4°C with antibody to phospho-phospholamban (Ser-16) (pPLN, Upstate #07-052) rabbit polyclonal Ab, using a 1:10,000 dilution in TBS-T containing BSA 5%. The next day, cells were washed with TBS-T; incubated at room temperature for 1 h with goat anti-rabbit Alexa Fluor 488-conjugated secondary Ab, using a 1:2000 dilution in TBS-T containing BSA 5%; washed with Triton X-100 0.2% in PBS, then with PBS; and finally mounted with Fluoromount G (Southern Biotech, AL).

Myocytes were examined by epifluorescence microscopy (E600, Eclipse; Nikon, Japan), equipped with a fluorescent filter cube (excitation 465 to 495 nm; dichroic mirror 505 nm; emission 515 to 555 nm, EF-4 FITC HYQ, Nikon) using a 10x and 40x objective. Fluorescent images of pPLN in 40-50 random fields were taken with a CCD digital camera (Spot Insight 2Meg FW Mono; Model # 18.0, Diagnostic Instruments, MI) and Spot Advanced software (Version 4.5). Capture time was adjusted to 1 s for all sample groups.

The intensity of pPLN fluorescence was analyzed in at least 113 rod-shaped myocytes in each group in each experiment, using ImageJ software (IJ 1.48v) (NIH). The average number of myocytes studied was 131 ± 10 for 23 separate analyses. In all experiments, the negative threshold was determined by examining fluorescence of the cells stained with nonspecific goat IgG as an isotype control antibody (Santa Cruz Biotechnology #sc-2028).

**pERK immunofluorescence in cultured NRVMs.**

pERK immunofluorescence in cultured NRVMs after A6, PMA, or vehicle for 5 min was quantified exactly as for AMVMs described above, except that myocytes were identified by staining with anti-total sarcomeric myosin heavy chain (MF20) mouse mAb, using a 1:200 dilution in TBS-T containing BSA 5%, and goat anti-mouse Cy3-conjugated secondary antibody (115-165-003; Jackson ImmunoResearch Lab, West Grove, PA), using a 1:400 dilution in TBS-T containing BSA 5%. MF20 imaging used fluorescent filter cube (EF-4 TRITC HYQ, Nikon), with excitation 540 to 525 nm; dichroic mirror 565 nm; emission 605 to 655 nm. The intensity of pERK fluorescence was analyzed in at least 200 cells positive for MF20 in 20-30 microscopic fields, using Quantity One software (Version 4.6.5; Bio-Rad, Hercules, CA). The average
number of myocytes studied was 331 ± 30 for 9 separate analyses.

**PKCδ activation in intact heart.**

Hearts from mice anaesthetized with ketamine (4 mg/g intraperitoneal) were perfused by the Langendorff method at a constant flow of 2-3 ml/min with modified Krebs Henseleit buffer containing (mmol/l): NaCl 138, KCl 4.7, MgSO4 1.2, glucose 10, pyruvate 10, CaCl2 2, HEPES 5, pH 7.2 at 37°C. After 10 min equilibration, hearts were perfused for 10 min with the β-AR antagonist timolol (2 μmol/l), then for 5 min with timolol alone, or timolol with A61603 (100 nmol/l, gift from Arthur Hancock, Abbott Lab, Abbott Park, IL), or PMA (100 nmol/L).

Ventricles were dissected quickly from perfused hearts, embedded immediately in tissue freezing medium (EMS, Washington, PA; #72592), frozen in 2-methylbutane pre-cooled by dry ice, and kept at -80°C until used to cut 8 μm sections with a cryostat (Jung Frigocut 2800E, Leica). Sections were dried 30 min at room temperature, fixed 10 min at -20°C in methanol:acetone (70:30, v:v), rinsed three times in PBS pH 7.4, incubated 1 h at room temperature with affinity purified goat anti-mouse IgG Fab fragment diluted 1:50 in PBS containing BSA 1% and Triton X-100 0.2%. Monoclonal PKCδ (BD Transduction Laboratories #P36520-610397, 1:25) was diluted in PBS containing BSA 3% and Triton X-100 0.2%, and incubated overnight at 4°C. Sections were washed twice for 3 min each with PBS containing Triton X-100 0.2%, then incubated 1 h at room temperature with Cyc3-conjugated secondary antibody (#115-165-003, Jackson ImmunoResearch Lab, 1:400 dilution in PBS with Triton X-100 0.2%). Slides were washed once, and nuclei were stained 5 min with bis-benzamide (10 mg/ml in PBS with Triton X-100 0.2%). Slides were washed twice with PBS with Triton X-100 0.2%, and once without Triton X-100. Coverslips were mounted with anti-fade medium (Prolong anti-fade kit, Molecular Probes, Eugene, OR; #P-7481). Slides were examined in a fluorescence microscope (Nikon Eclipse E600), and photographed with a digital camera (Spot, Diagnostic Instruments).

**CONTRACTION ASSAY.**

**Contraction in isolated AMVMs**

AMVMs were maintained for no longer than 4-5 h at room temperature after isolation in buffer containing (mmol/l): NaCl 120, KCl 4.7, MgSO4 1.2, Na2HPO4 0.6, KH2PO4 0.6, glucose 5.5, HEPES 10, BDM 10, Ca2+ 0.6, and calf serum 10%, at pH 7.0. To measure contraction, an aliquot of cells was placed on an inverted microscope in a chamber (Model FHD, Ionoptix, Milton MA) superfused with a Krebs Henseleit solution oxygenated with 95% O2/5% CO2 to give a pH 7.4 at room temperature, and containing (mmol/l): NaCl 112, KC1 5, MgCl2 1.2, glucose 10, NaHCO3 24, Na2SO4 1.2, NaH2PO4 2.0, and CaCl2 1. Myocytes were paced electrically at 0.5 Hz using supramaximal stimuli (4 ms square wave pulses). A video image of the myocyte using a 40x objective was used to monitor contraction continuously from changes in sarcomere length (Ionoptix, Milton, MA). Sarcomere length of rod-shaped myocytes was measured using a rectangular measurement window positioned toward the center of the cell, spanning approximately two-thirds of the cell length, and about half of the cell width.

Stimulated myocytes were equilibrated for approximately 10 min until contraction was stable. Contraction was then recorded continuously; superfusion with the α1A agonist A61603 or the β agonist isoproterenol or vehicle was initiated after 200 s; and the change in contraction was assessed after 300 s for A61603 or 200-300 sec for isoproterenol. Some myocytes were
pretreated with PTX as above for pPLN (0.3 μg/ml for 3 h at 37°C).
Myocytes were identified as responding to A61603 if the contraction amplitude changed by more than the median ± range of the value obtained using vehicle alone.

**RADIOLIGAND BINDING.**

**α1- and β-AR radioligand binding in hearts.**

For whole heart membrane preparation, hearts were harvested and rinsed in ice cold PBS Ca++ and Mg++ free (UCSF Cell Culture Facility, Code: CCFAL004-155D01), then homogenized in 4 ml buffer: 0.25 M sucrose(#S0389), 30 mM histidine (#H8000) 1.0 mM EDTA (#E5134), and 0.1 mM PMSF (#P7626), made up in 100% EtOH, all from Sigma-Aldrich, St. Louis, using the Kinematica Polytron, PT 10/35 GT, #13-874-617 (Fisher Scientific, Waltham MA) on ice for 15 sec at setting 6.5. The homogenate was transferred to 12 ml centrifuge tubes, balanced to 12 grams, and centrifuged at 26,000 rpm (100,000 x g) for 45 min in a Beckman SW41 Ti rotor using a L8-80M Ultracentrifuge (Beckman Coulter, Indianapolis IN). The pellet was resuspended in buffer, homogenized, and spun again in the same manner.

The resulting pellet was suspended in one of 2 binding buffers: for α1-ARs, Tris 50 mmol/l pH 7.4 and EDTA 1 mmol/l, or for β-ARs Tris 20 mmol/l pH 7.4, NaCl 154 mmol/l, and MgCl2 5 mmol/l, and sonicated using a ultrasonicator #W-380 (Heat Systems Ultrasonics, Farmdale NY) set at 50% Duty Cycle, Output Control 4 with 5X 5 second bursts on ice. Membrane protein concentration was determined using Bradford Protein Assay #5000006 (Bio-Rad, Hercules California), and protein concentrations were set to 1 mg/ml.

α1-AR saturation binding for 1 h at 30°C used 200 μg membrane protein per tube in 1 ml buffer with 6 concentrations (0.04-1.2 nmol/l) in triplicate of 3H-prazosin (#NET-823, Perkin Elmer, Waltham MA), phentolamine (10 μmol/l, #P7561 Sigma-Aldrich) was used to define non-specific binding. β-AR saturation binding for 90 min at 25°C used 50 μg protein per tube in 0.25 ml buffer with 6 concentrations (0.04-1.0 nmol/l) in triplicate of 125I-cyanopindolol (#NEX189, PerkinElmer, Waltham MA), L-propranolol (1 μmol/l, Sigma-Aldrich #P8688) was used to define non-specific binding.

Bound ligand was separated from free ligand by filtration with ice-cold assay buffer through glass fiber filters, rinsed 4 times with 4ml cold assay buffer, dried and counted. Total receptor number (Bmax) and binding affinity (Kd) were calculated by nonlinear regression with GraphPad Prism (Version5.0d; GraphPad Software).

**α1- and β-AR radioligand binding in isolated AMVMs.**

Isolated AMVMs were counted, pelleted, and suspended in one of 2 binding buffers: for α1-ARs, Tris 50 mmol/l pH 7.4 and EDTA 1 mmol/l, or for β-ARs Tris 20 mmol/l pH 7.4, NaCl 154 mmol/l, and MgCl2 5 mmol/l. Cells were lysed using an ultrasonicator #W-380 (Heat Systems Ultrasonics, Farmdale NY) set at 50% Duty Cycle, Output Control 4 with 5X 5 sec burst on Ice. For α1-AR binding, cell numbers were set to 225,000 myocytes/ml, and α1-AR saturation binding for 1 h at 30°C used 200 μl lysed cells, equal to 45,000 myocytes per tube in 1 ml buffer with 6 concentrations (0.04-1.2 nmol/l) in triplicate of 3H-prazosin (#NET-823, Perkin Elmer, Waltham, MA), phentolamine (10 μmol/l, Sigma-Aldrich #P7561) was used to define non-specific binding.

For β-AR saturation binding, cell numbers were set to 30,000 myocytes/ml. Binding for 90 min at 25°C used 100 μl lysed cells, equal to 6,000 myocytes per tube in 0.25 ml buffer with 6 concentrations (0.04-1.0 nmol/l) in triplicate of 125I-cyanopindolol (#NEX189, PerkinElmer,
Waltham MA), L-propranolol (1 μmol/l, Sigma-Aldrich #P8688) was used to define non-specific binding.

α1-AR subtype proteins were quantified by competition for binding of ³H-prazosin (0.5 nmol/l) with 22 concentrations (0.05 nmol/l to 500 μmol/l) in duplicate of 5-methylurapidil (#U101, Sigma-Aldrich), an α1A-selective antagonist. β-AR subtype proteins were quantified by competition for binding of ²⁵I-CYP (50 pmol/l) with 22 concentrations (50 pmol/l to 500 μmol/l) in duplicate of ICI-118,551 (#I127, Sigma-Aldrich), a β2-selective antagonist.

Bound ligand was separated from free ligand by filtration with ice-cold assay buffer through glass fiber filters, rinsed 4 times with 4ml cold assay buffer, dried and counted. Total receptor number (Bmax) and binding affinity (Kd) were calculated by nonlinear regression with GraphPad Prism (Version 5.0d; GraphPad Software).

**β-AR radioligand binding in isolated AMVM and NMC crude membranes.**

Isolated AMVMs, or NMCs from 2-4 hearts, were pelleted and suspended in Tris 20 mmol/l pH 7.4, NaCl 154 mmol/l, and MgCl₂ 5 mmol/l. Cells were lysed as above. Binding for 90 min at 25°C used crude membranes at 50 μg protein (Bradford) per assay tube in 0.25 ml buffer with 6 concentrations (0.04-1.0 nmol/l) in duplicate of ¹²⁵I-cyanopindolol (#NEX189, PerkinElmer, Waltham MA), L-propranolol (1 μmol/l, Sigma-Aldrich #P8688) was used to define non-specific binding.

**DATA ANALYSIS.**

Results are presented as mean ± SE. Significant differences (p<0.05) were tested using one-way ANOVA with Newman-Keuls multiple comparison post-test (GraphPad Prism version 5.0d). Distribution was examined using D’Agostino and Pearson omnibus normality test. Most values for isolated cell contractility and immunohistochemistry were not distributed normally, and we used the median ± range to define values above or below the control. Linear regression tested for associations. GraphPad Prism fitted concentration-response and radioligand binding data.
SUPPLEMENTAL REFERENCES


5. Lobe CP, Guo C. Reporter genes to detect cre excision in mice. *Curr Protoc Toxicol*. 2002;Chapter 15:Unit15 14


