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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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Abstract

Rationale—It is unknown if every ventricular myocyte expresses all 5 of the cardiac adrenergic receptors (ARs), beta-1, beta-2, beta-3, alpha-1A, and alpha-1B. The beta-1 and beta-2 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 alpha-1A and alpha-1B knockin reporters, and beta-1 and beta-2 knockout mice. Using individual isolated cells, we measured knockin reporters, mRNAs, signaling (phosphorylation of ERK and phospholamban), and contraction. We found that the beta-1 and alpha-1B were present in all myocytes. The alpha-1A was present in 60%, with high levels in 20%. The beta-2 and beta-3 were detected in only about 5% of myocytes, mostly in different cells. In intact heart, 30% of total beta-ARs were beta-2 and 20% were beta-3, both mainly in nonmyocytes.

Conclusion—The dominant ventricular myocyte ARs present in all cells are the beta-1 and alpha-1B. The beta-2 and beta-3 are mostly absent in myocytes but are abundant in nonmyocytes. The alpha-1A is in just over half of cells, but only 20% have high levels. Four distinct myocyte AR phenotypes are defined: 30% of cells with beta-1 and alpha-1B only; 60% that also have the alpha-1A; and 5% each that also have the beta-2 or beta-3. 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.

Keywords

Receptors; adrenergic; beta; receptors; adrenergic; alpha; cardiac myocyte; adrenergic receptor

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DISCLOSURES

None.

Subject Terms

Autonomic Nervous System; Cell Biology/Structural Biology; Cell Signaling/Signal Transduction; Myocardial Biology; Basic Science Research

INTRODUCTION

The heart has five 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 α1 and β3.[1,2] 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.[5] However, very 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 (AKO) reporter mouse, with bacterial β-galactosidase (bGal) replacing exactly the 1st coding exon, to show that α1A expression in the abdominal arteries was markedly heterogeneous,[6] 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. We measured in individual wild type (WT) myocytes mRNAs, signaling, and contraction. β-AR subtypes were deduced using β1- and β2-KO myocytes, and receptor levels were quantified by radioligand binding.

Surprisingly, we find that the dominant myocyte ARs are the β1 and α1B, which are present in all cells. The α1A is expressed and functional in a 60% subset, with 20% having high receptor levels. The β2 and β3 are mostly absent on myocytes, but abundant on nonmyocytes. These data revise concepts of cardiac adrenergic signaling mechanisms. The results also raise cautions in experimental design, such as receptor overexpression in myocytes that do not express the AR normally.

METHODS

Mice were primarily adult males in the C57Bl/6J background. α1A-KO reporter mice have bGal replacing exactly the first coding exon.[6] 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 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. Mice in PKCδ experiments were in FVBN/129 mixed background. Adult mouse ventricular myocytes (AMVMs) were isolated by perfusion with collagenase; RV, septum, and LV were dissociated separately in some experiments. Sprague Dawley neonatal rat ventricular myocytes (NRVMs) were isolated with trypsin. AMVMs 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 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 dually phosphorylated on tyrosine and threonine (pERK) was detected in cultured myocytes by immunoblot or immunocytochemistry with a rabbit monoclonal antibody (Ab, Cell Signaling #4370); and phospholamban phosphorylated on serine 16 (pPLN), with a rabbit polyclonal Ab (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 mAb (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 ^{3}H-prazosin for α1-ARs and ^{125}I-cyanopindolol (CYP) for β-ARs. The fraction of AR subtypes was determined using competition binding in WT and binding in KOs.

Results are mean ± SE. GraphPad Prism v5.0d was used to test for a normal distribution; for significant differences (p<0.05), using t-test or one-way ANOVA and Newman-Keuls post-test for more than 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 (r2=0.94, p<0.001), validating bGal as a surrogate for α1A transcription.

In the intact AKO heart, bGal was quite heterogeneous, with stain intensity from none to high, as visualized by conventional histochemistry or by optical projection tomography (Figure 1A and Online Movies). This heterogeneity was seen also 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 (≥7) (Online Figure IB). Overall, we counted dots in ~16,000 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 (≥7 dots). Selection bias was not a confounder, since identical numbers of myocytes were isolated from AKO and WT ventricles (AKO $2.1\pm0.07\times10^6$, N=33 hearts vs. WT $2.1\pm0.05\times10^6$, 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, vs. 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.[7] 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 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 (see Figure 5). Interestingly, there was no correlation between levels of the α1A and α1B in individual myocytes (Figure 2B). All cells studied had α-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-Abs to test for α1A protein, since commercial α1-Abs are nonspecific for any α1-subtype or for total α1-ARs.[8] 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.[9] We used the highly selective and potent α1A agonist, A61603,[10] and

quantified maximum ERK activation in individual cells using immunofluorescence with an Ab for dually phosphorylated ERK1/2.

Figure 3A shows an experiment in which 1 of 2 A6-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, since values were not distributed normally. In the same experiment, 93% of PMA-treated cells had pERK above this level, as a positive control, and none of A61603-treated AKO cells, 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 200–250 myocytes, indicating that A61603 activated ERK in 62±2% of WT myocytes, significantly more than vehicle and less than PMA, and that A61603 was inactive in AKO myocytes (n=5 independent experiments).

In summary, A61603 activates ERK in 62% of WT myocytes, with 20% activating ERK to the same high extent as seen with the PKC activator PMA.

The α1A mediates contraction in a subset of myocytes, the α1B in all cells

As a second endpoint for 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.[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 (PIE) or a negative inotropic effect (NIE), as observed previously.[12] A61603-responders and A61603-nonresponders contracted equally with ISO (see 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 (PE) (below). Combining WT cells with either a PIE or an NIE, 21% had a high response to A61603, defined as ≥40% change in contraction (Figure 4B).

To test for function of the α1B, we treated AKO myocytes, which have only the α1B,[13] with the nonselective α1-agonist PE (10 μM) in the presence of the β-antagonist timolol (10 μM). PE in the presence of timolol stimulated contraction in 14 of 14 AKO myocytes with a predominant NIE (79%) and varying amplitude. Vehicle did not change contraction in these experiments (6 of 6 cells).

In summary, A61603 activates contraction in 64% of WT myocytes, with 21% having a high level of activation, either negative or positive. The α1B is functional in all cells.

The α1A in a subset of myocytes in the intact heart and in the rat

To test the generality of α1A presence in a myocyte subset, we studied the intact mouse heart and cultured neonatal rat ventricular myocytes (NRVMs). A61603 infusion in the perfused mouse heart stimulated nuclear translocation of PKCδ in a small subset of myocytes, in contrast with PMA, which increased nuclear PKCδ in all cells (Online Figure III). PKCδ is a known target for α1-ARs and PMA in myocytes.[14] In NRVMs, A61603 activated ERK in 67±2% of myocytes, compared with 90±1% activation by PMA (Online Figure IV). Together, these data showed that α1A function in a myocyte subset is not limited to mouse myocytes in vitro, but is seen in the intact mouse heart and in cultured rat myocytes.

β1 mRNA is in all myocytes, β2 and β3 mRNA and binding are mostly absent in myocytes

The microarrays used for the other AR mRNAs did not contain probes for the β1-AR, so we quantified β1-AR mRNA using RT-qPCR in single isolated myocytes, and compared levels of the α1A mRNA in the same cells. β1-KO and α1A-KO myocytes were negative controls to identify cells that expressed an mRNA. Figure 5A shows that all of 18 WT myocytes had β1 mRNA, as well as two of two α1A-KO myocytes. In the same 18 WT myocytes, α1A mRNA was present in only 9 (50%), with 4 of 18 (22%) having high levels, and in 1 of 2 β1-KO cells. Interestingly, cells with high levels of α1A mRNA tended to also have high levels of β1 mRNA (Figure 5B). Since α1A and α1B mRNA levels are not correlated (Figure 2B), it is unlikely that the positive relationship between α1A and β1 mRNA levels is simply a reflection of overall AR levels. Also notable, the range of α1A mRNA levels was about 100-fold from lowest to highest, vs. 30-fold for β1 mRNA (Figure 5B).

We measured β2 and β3 mRNA levels in each of 50 WT myocytes using microarray, as with the α1A and α1B mRNAs (Figure 2).[7] As before, a cell was defined as positive if the mean ± SE mRNA level from the 3 to 6 probes on the array for each mRNA was greater than the background level defined by 5000 probes directed to nonsense sequences. Surprisingly, by this criterion, only 4 of 50 myocytes (8%) were positive for β2 or β3 mRNA (Figure 6A). Interestingly, plotting β2 and β3 mRNAs vs. 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 about 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, since 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 β1A. 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 vehicle-treated cells. The experiment in Figure 7A right shows that ISO activated PLN in 97% of WT myocytes, vs. 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 vs. 3±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 very 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 theβ3 is not functional in myocytes.

The β1-AR stimulates contraction in all myocytes, the β2 and β3 do not stimulate contraction

As a second endpoint 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 PIE with ISO, vs. none of 10 β1-KO myocytes, pretreated with PTX to enhance any contractile response through the β2-AR.[23,24,26] ISO stimulated contraction in 2 of 20 β1/2-KO cells, but these responses were only about 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, approximately 60% have the α1A, and only about 5% each have the β2 and β3. An average about 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 very 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 very 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 β1A expression and function. These findings suggest a reevaluation of cardiac adrenergic signaling models and experimental approaches, as discussed below.

Because valid AR Abs 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, e.g. 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; prior 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 about 80 nmol/l would lose selectivity for the β2, and most studies use dosing well in excess of this.[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-118,551, 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-118,551 over about 30 nmol/l would lose selectivity for the β2-AR, and most studies use higher dosing.[32–34,37,41,44] In summary, nonselective zinterol and ICI-118,551 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,[48] that has uncertain specificity,[21,28] and by the agonist BRL37344,[49] that is not selective for the β3, or even for ARs.[50–52]

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,[53] and another finds 17% β2.[33] 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.[54] This study used single-point binding assays,[54,55] which might explain the difference from our finding of no detectable binding in the β1-KO (Figure 6). Also surprising, we find no prior 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,[1,2,5] comprising about 20% of total β-ARs (Figure 6), most likely in endothelial cells (Online Figure V).[27]

Another group has extensive evidence for β2 and β3 signaling and contraction effects in β-AR KO neonatal mouse myocytes.[23,24,26,56] These results do not prove necessarily that the

β2 and/or β3 are in a high percent of neonatal myocytes, since the studies are done in uniformly beating cell syncytia, where a few cells can drive contraction, and/or β2 or β3 stimulation of contaminating nonmyocytes can secrete paracrine factors for myocytes.[57] 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, since we studied cells from mice aged about 8–20 weeks. Furthermore, transcript variability in individual cells is deterministic, not stochastic,[58] 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[59,60] or novel signaling.[54] Second, β2, β3, and β1A electrophysiology and EC 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, e.g. group data for the α1A contraction data in Figure 4 would be very 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.[61] In myocytes, for example, α1-ARs in cultured myocytes induce β-MyHC.[62] 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.[63] 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 β2 as believed currently. Furthermore, since 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. Very 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, since there are no agonists selective of 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.[64] Since NE has about 10-fold lower affinity for the α1B vs. the β1,[65] an α1B-β1 interaction could be especially relevant in heart failure, when β1-ARs are down-regulated 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 down-regulated in heart failure.[27,66] Thus, maintenance or increase of β2 and β3 levels in heart failure is likely due to nonmyocyte proliferation, not regulation different from the down-regulated 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 (review in[4]). In this regard, some studies using β2-KO models indicate that β2 activation can be toxic, not protective as generally thought.[67,68] Increased β2 effects in nonmyocytes, such as fibroblast proliferation, could explain maladaptive effects of β2 activation.

Third, the α1A stimulates inotropy and protection throughout the whole heart,[69,70] and a key question for further study is the mechanism, when the α1A is present in only 60% of myocytes, and at high levels in only 20% (Figure 9). The α1A is absent in nonmyocytes,[17,71] so a nonmyocyte-to-myocyte mechanism similar to β2 or β3 effects is not relevant. Presumably the α1A 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 wild type β-myosin among individual myocytes.[72] Accordingly, it will be important to develop new methods to phenotype individual myocytes and relate phenotypes to receptor expression.[73]

In summary, the β1 and α1B are the dominant cardiac myocyte ARs, present in all cells, where the α1B might serve as a brake on β1 inotropic effects. The β2 and β3 are each in only about 5% of myocytes, but both are abundant in nonmyocytes, a distribution that enlightens several aspects of β2 and β3 biology. The α1A is in a 60% subpopulation, and present at high levels in only 20% of myocytes. Cardioprotective and inotropic signaling by the α1A would appear to involve myocyte-to-myocyte mechanisms. The results define myocytes with 4 distinct AR phenotypes, which are likely to determine distinct downstream myocyte phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

A6, A61603 (*N*-[5-(4,5-dihydro-1*H*-imidazol-2-yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfo

an α1A-AR selective agonist

α1-Ars
alpha-1-adrenergic receptors

α1BKO
α1B-AR knockout

α-MyHC
alpha-myosin heavy chain

Ab
antibody

AKO
α1A-AR knockout

Alk phos
alkaline phosphatase

AMVM
adult mouse ventricular myocyte

bGal
β-galactosidase

Cq
quantification cycle

CYP
cyanopindolol

hPLAP
human placental alkaline phosphatase

ISO
isoproterenol

LVFW
LV free wall

MyHC
myosin heavy chain

NIE
negative inotropic effect

NR
no response

NRVM
neonatal rat ventricular myocyte

pERK
phosphorylated-ERK

PIE
positive inotropic effect

pPLN
phosphorylated phospholamban

PMA
phorbol myristate acetate

RT-qPCR
reverse transcription quantitative real time polymerase chain reaction

RVFW
RV free wall

VEH
vehicle

WT
wild type

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NOVELTY AND SIGNIFICANCE

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 α1B-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.

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 α1B 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 sympathetic responses, not the β1 and β2 as believed. 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, 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.

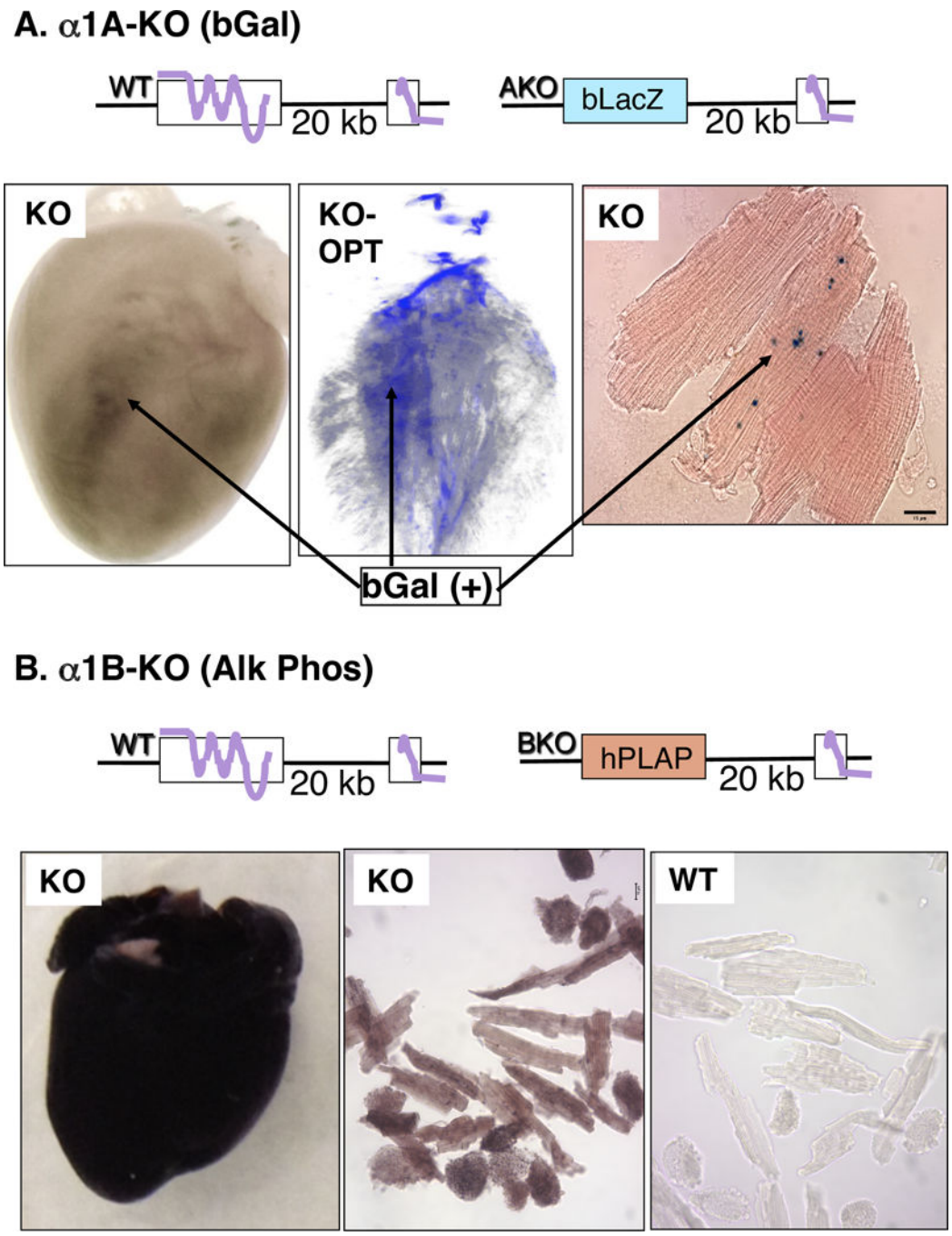


Figure 1. Knockin reporter mice show the a1A in a myocyte subset, the a1B in all cells

Knockin replaced the 1st coding exon with bacterial Lac Z (α1A) or human placental alkaline phosphatase (hPLAP) (α1B). **A. α1A-KO with bGal stain**. Homozygous adult AKO hearts were stained with Bluo-Gal by Langendorff perfusion (**left**), then cleared and scanned by Bioptonics OPT (**middle**); the image is from a QuickTime movie (Online). Cells (**right**) were stained after isolation. bGal positive heart areas and one positive cell (with blue dots) are indicated. **B. α1B-KO with alk phos stain**. Heterozygous adult BKO hearts were stained by Langendorff perfusion with X-Phos/NBT for alk phos, after heat inactivation of

endogenous alk phos (**left**). Cells (**middle and right**) were stained after isolation. All KO cells are positive for alk phos.

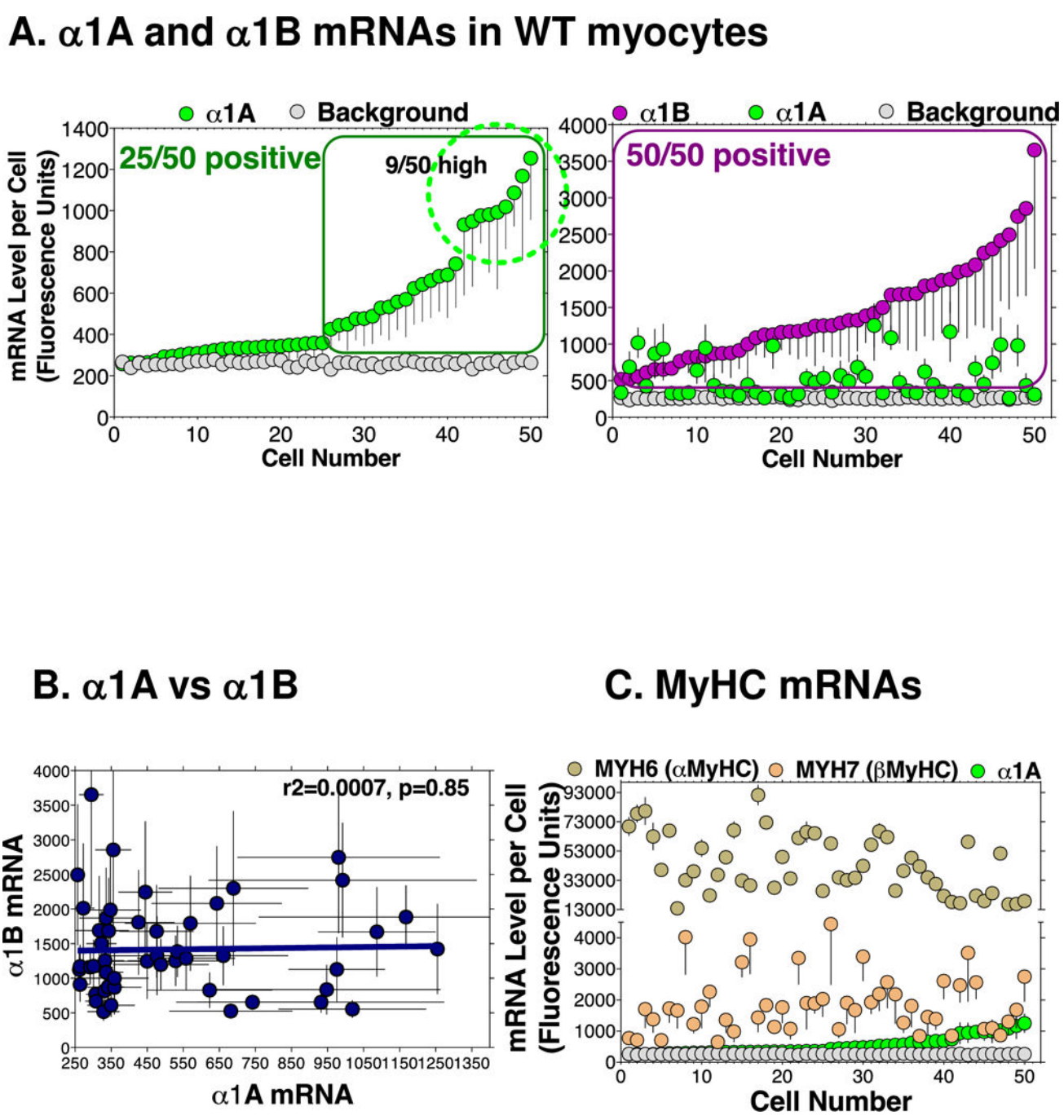


Figure 2. The a1A is expressed in a subset of isolated WT myocytes, the a1B is present in all cells

α1A, α1B, αMyHC, and βMyHC mRNAs were quantified on microarrays in each of 50 individual WT adult mouse ventricular myocytes. Each point is 1 myocyte, and values are mean±SE of 6 different probes for each mRNA on the array (3 probes for α1B, and 5000 probes for Background, grey). **A**. Cell data are sorted for increasing levels of a1A mRNA (**left**, green) or increasing levels of a1B mRNA (**right**, purple). Twenty five of 50 cells have α1A mRNA with mean-SE above background, whereas all cells have α1B above background. **B**. α1A levels are plotted against α1B levels in the same cells; there is no

correlation. **C**. aMyHC and bMyHC mRNA levels in the same myocytes, sorted for increasing levels of a1A mRNA. All cells express both MyHC mRNAs; note the interrupted Y-axis. There is a negative correlation between a1A and aMyHC levels (r2=0.25, p=0003), but no correlation with βMyHC levels (r2=0006, p=0.87). α1B levels did not correlate with bMyHC mRNA (r2=0.02, p=0.27) or aMyHC (r2=0.04, p-0.16) (not shown).

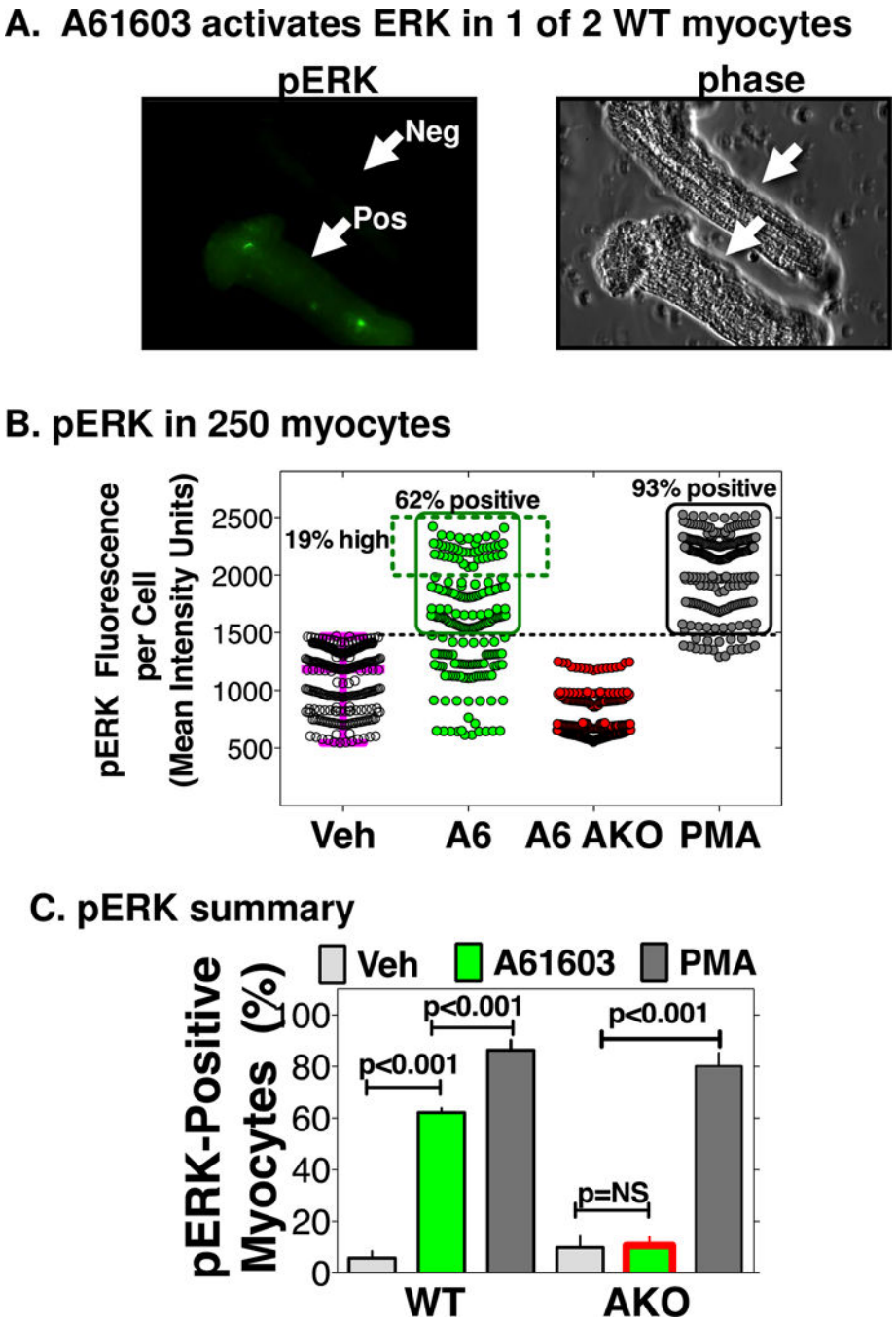
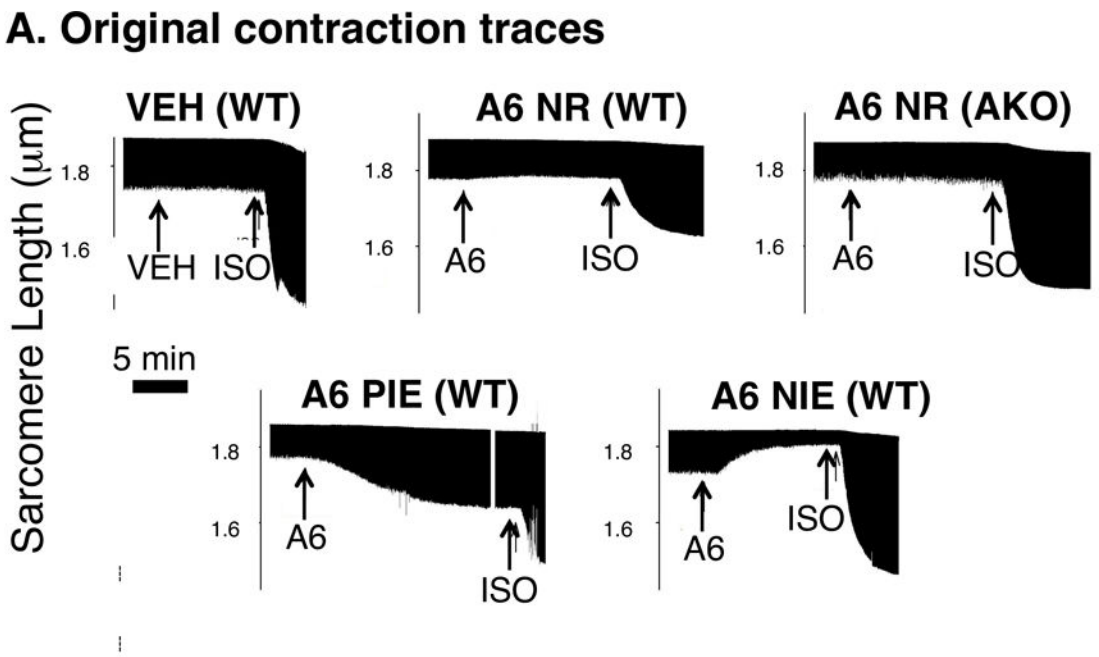
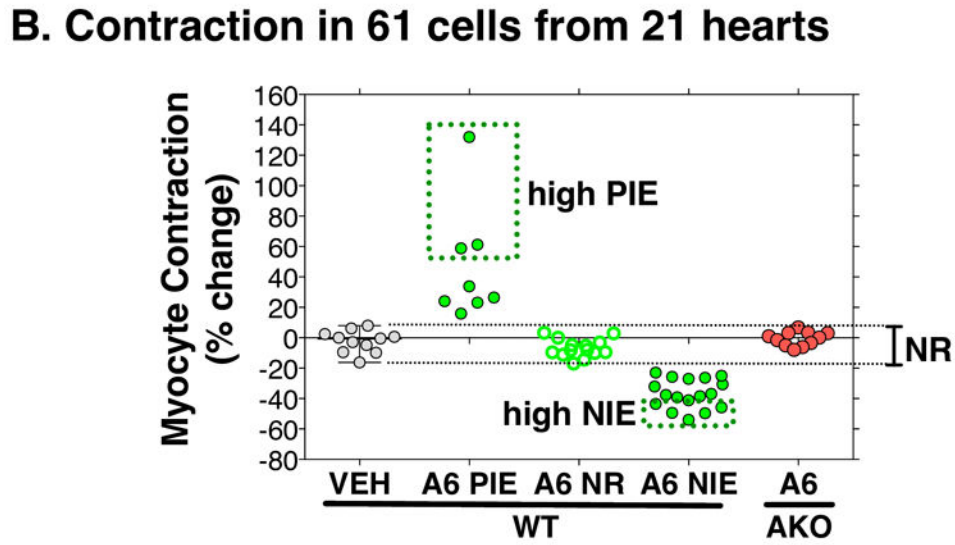


Figure 3. The a1A functions in a subset of WT myocytes to activate ERK

Cultured adult mouse myocytes were treated with agonists or vehicle for 5 min for maximum ERK activation, then fixed, and stained for phosphorylated (activated) ERK. **A**. The α1A agonist A61603 (200 nmol/l) activates ERK in 1 cell (**left**, "Pos" green fluorescence) of the 2 shown (**right**, phase). Original 100x. **B**. pERK mean fluorescence units per myocyte (units/cell area) was quantified in ~250 individual WT cells after treatment with A61603 or PMA (100 nmol/l) as a positive control. α1A-KO cells treated with A61603 are a negative control. Each dot is one 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 fluorescence was counted in ≥200 rod-shaped myocytes in each of 5 experiments with different WT and AKO hearts; bars are mean±SE, with p by ANOVA and Newman-Keuls post-test. A61603 activates ERK in 62±2% of WT cells, vs. 6±3% in vehicle, and PMA activates ERK in 86±4%. A61603 has no effect in AKO myocytes, and PMA shows that AKO cells are viable and have ERK that can be activated.





A6 Summary	Studied	R	NR	PIE	PIE>40%	NIE	NIE>40%
# WT Cells	39	25	14	8	3	17	5
% Cells	100%	64%	36%	20%	8%	44%	13%

Figure 4. The a1A functions in a subset of WT myocytes to activate contraction

Sarcomere length (SL) was measured in paced myocytes treated with Vehicle (VEH), A61603 (A6, 100 nmol/l), and the b1/2-AR agonist isoproterenol (ISO, 1 mmol/l). **A**. Raw tracings of SL vs. time, with VEH, A6, and ISO added at arrows; NR=No Response to A6; PIE=Positive Inotropic Effect; NIE=Negative Inotropic Effect. **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 one 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 panel B). Every tested cell responded to ISO (Figure 7).

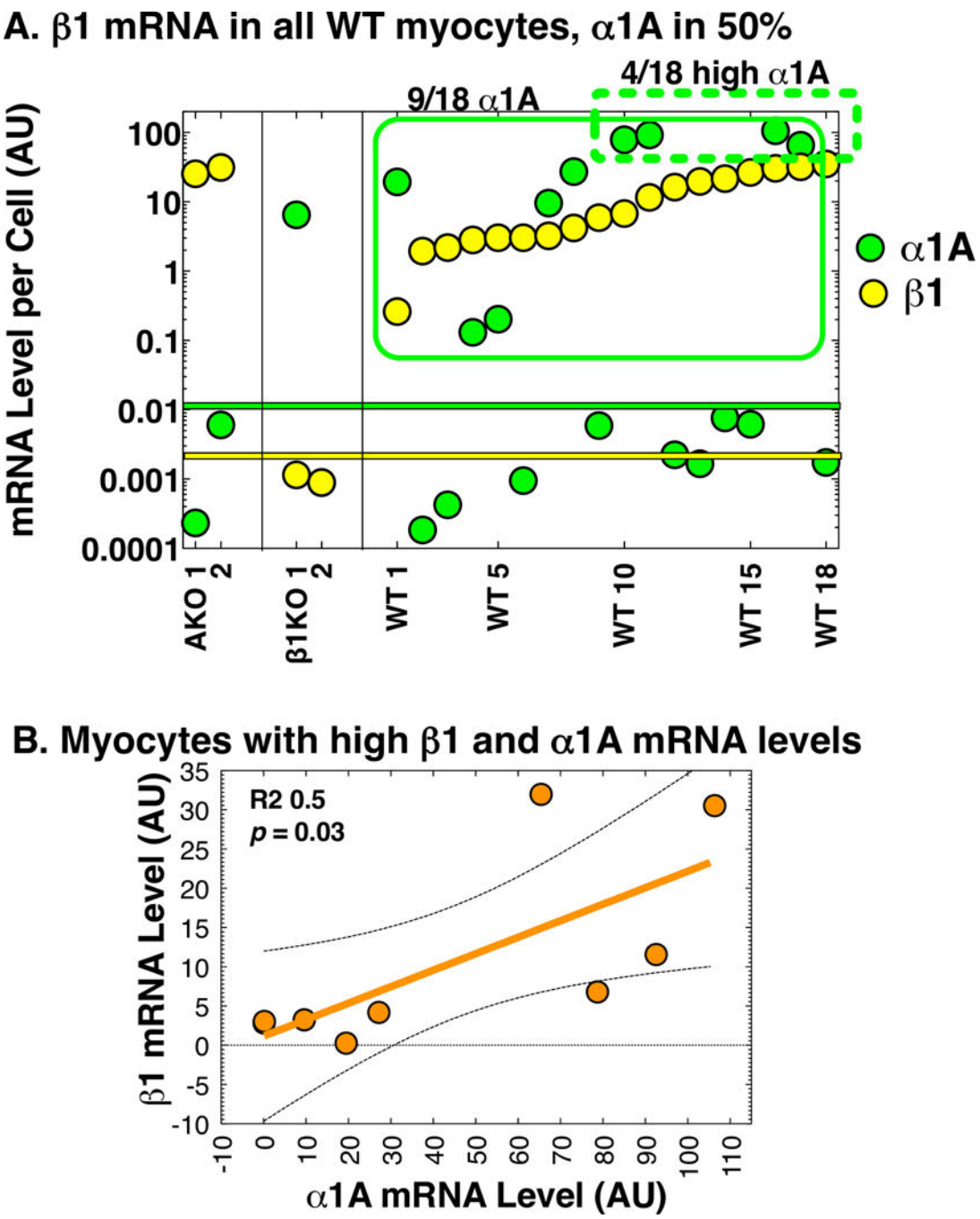


Figure 5. b1 mRNA is in all cells, α1A mRNA is in a subpopulation of β1 cells

A. Single cell RT-qPCR quantified β1 and α1A mRNAs in 18 WT myocytes. The highest levels in 2 KO myocytes for each receptor (left of graph) were used to define negative expression (yellow horizontal line for β1 and green line for α1A). Each point is one myocyte; values are arbitrary units (AU) relative to β-actin and β2-microglobulin; note log scale. **B**. α1A levels are plotted against β1 levels in the same WT cells. Cells with high α1A tended to have high β1. Data from panel A; dotted lines are 95% confidence limits.

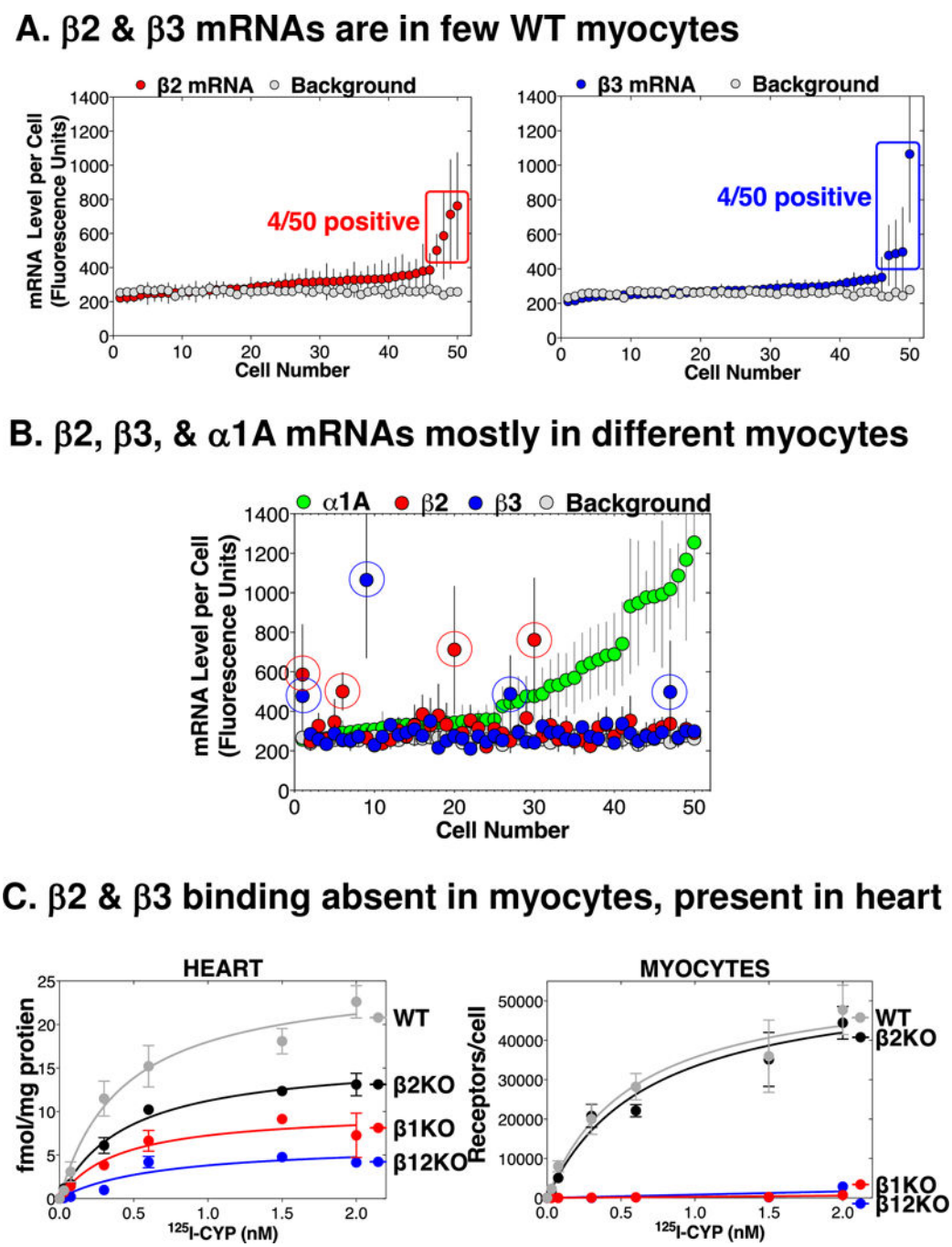


Figure 6. The b2- and b3-ARs are expressed in a very small fraction of ventricular myocytes

A. b2 and b3 mRNAs were quantified on microarrays in each of 50 individual WT AMVMs; values (b2 red, b3 blue) are mean ± SE of 3 (b2) or 6 (b3) different probes on the array (5000 probes for Background, grey). Each dot=1 myocyte. **B**. The mRNA values in the same cells are sorted by increasing a1A mRNA levels (green, from Figure 2); circles are cells positive for b2 and b3. **C**. b-AR proteins were quantified by ^{125}I-CYP binding in WT and KO whole heart or isolated myocytes (b1, b2, and b1b2 KOs). Saturation binding curves

show mean ± SE specific binding from 3 hearts of each genotype. Use of 3-fold higher [^{125}I-CYP] did not detect b3-ARs in the b1b2 KO myocytes (not shown).

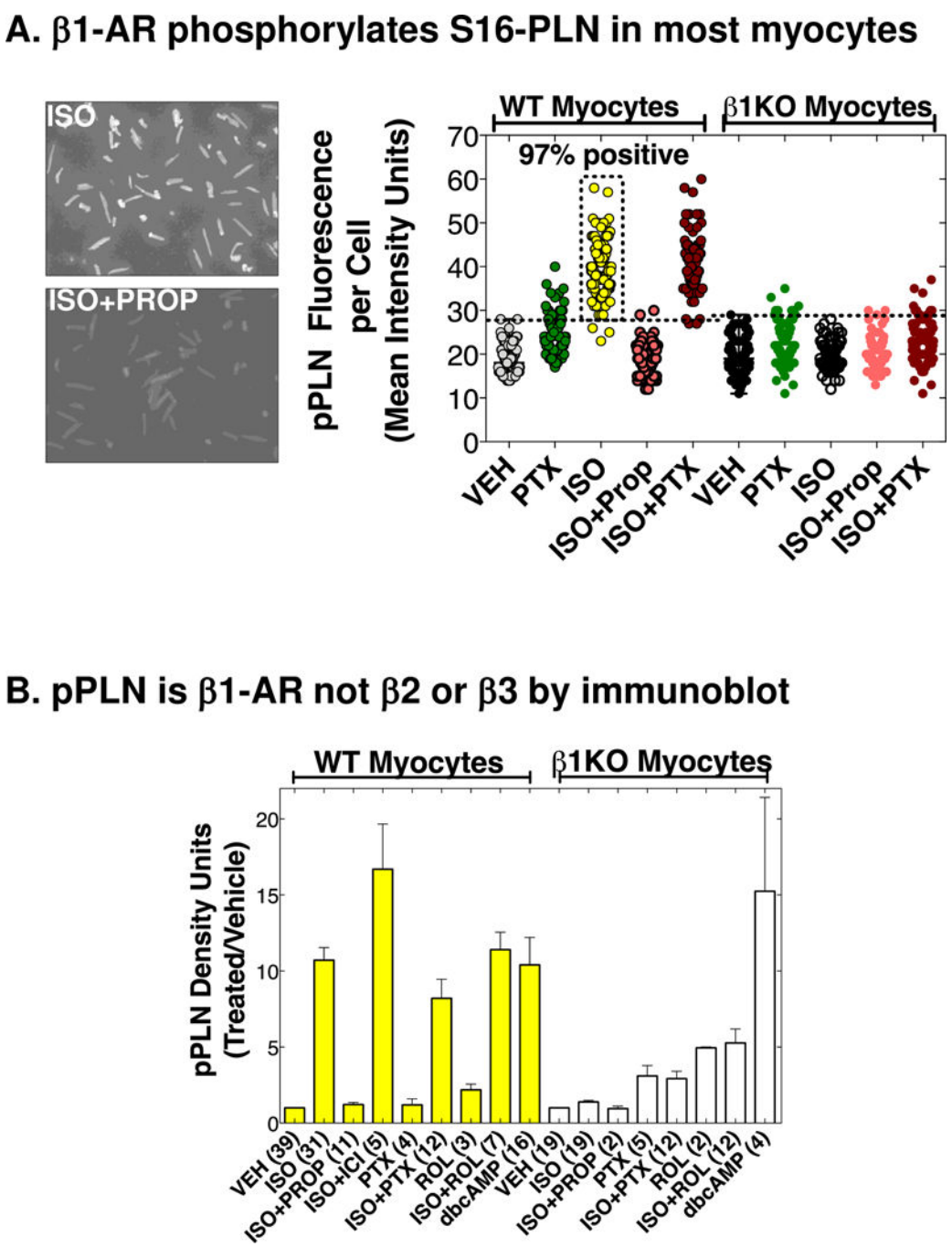
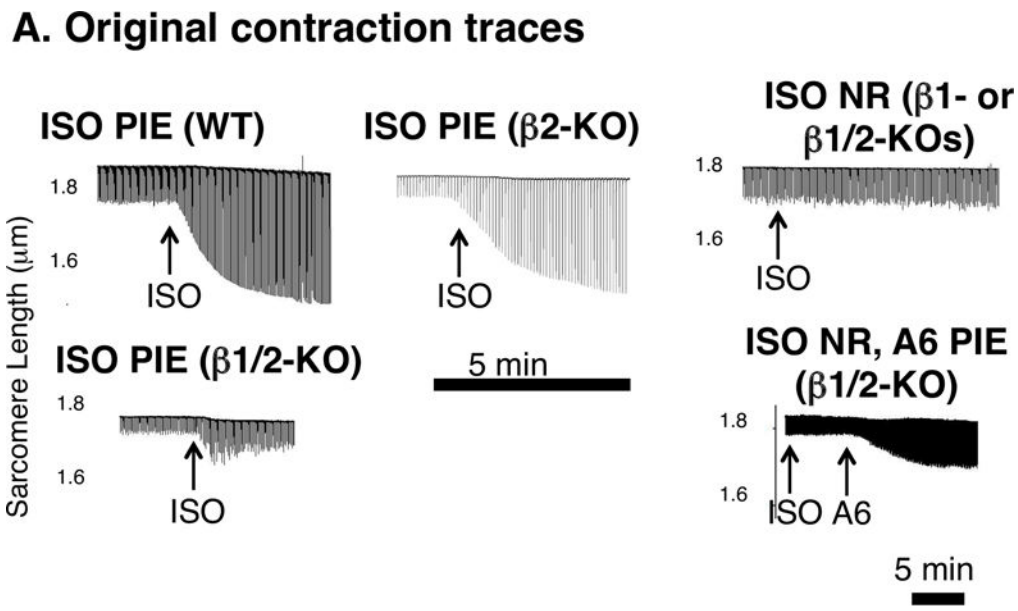


Figure 7. The β1-AR functions to phosphorylate S16-PLN in most myocytes, b2 and/or b3 are inactive

Cultured adult mouse myocytes were treated with ISO or vehicle (ascorbic acid 100 mmol/l) for 5 min, when S16-phosphorylated PLN (pPLN) was maximum. **A**. WT or β1-AR KO myocytes had ISO 10 nmol/l without or with PROP 1 mmol/l; pretreatment with pertussis toxin (PTX, 0.3 μg/ml for 3 h) inactivated Gi. **Left**, fluorescent image, original 10X; **Right**, each dot is 1 cell, with ~115 cells/group; dotted line indicates median+range of vehicle group. ISO does not increase the percent cells with pPLN in the β1KO. **B**. Cultured WT or

β1KO myocytes were treated with ISO (10 nM-1 μM), L-propranolol (PROP, 1 μM), ICI 118,551 (50 nM, a concentration ~5-fold the IC50 at the b2-AR), or the cell permeable cAMP analog dibutyryl cAMP (dbcAMP, 5 mmol/l), the phosphodiesterase 4 inhibitor rolipram (ROL, 1 μM), or vehicle; some cells were pretreated with pertussis toxin (PTX).



B. Summary responses

Genotype	Cells Studied	PTX-Treated	Cells Responding	% Responders
WT	40	2	40	100
β2-KO	28	0	28	100
β1-KO	10	10	0	0
β1/2-KO	20	0	2	10

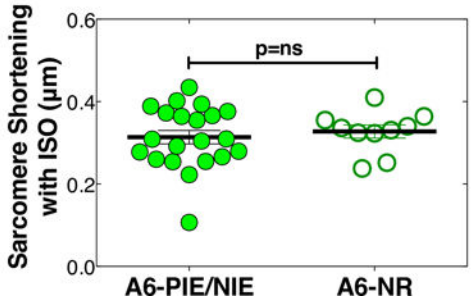


Figure 8. b2- and b3-ARs are functional in a very small fraction of ventricular myocytes, β1-ARs function in all cells

Sarcomere length (SL) was measured in myocytes paced at 0.5Hz and superfused at room temperature in oxygenated Krebs buffer with isoproterenol (ISO, 1 mmol/l; 10 nmol/l for β2-KO cells). ISO was added at arrows; A61603 (100 nmol/l) was tested after ISO in some cells, others were pretreated with PTX. Cell genotypes are noted. NR=No Response; PIE=Positive Inotropic Effect; NIE=Negative Inotropic Effect. **A**. Examples of contraction traces. The time scale at bottom right applies only to the contraction with A61603. **B**.

Summary of responses. **C**. ISO responses in cells that did or did not respond to A61603 (from Figure 4).

A. Summary percent myocytes positive for each AR

			% Myocytes Positive					
Myocyte Assay	**Genotype**	**Agonist**	**α1A**	**α1A high**	**α1B**	**β1**	**β2**	**β3**
Knockin reporter	KO	none	61	18	100			
mRNA	WT	none	50	20	100	100	8	8
pERK	WT	A6	62	20				
pERK	NRVM	A6	67					
pPLB	WT & KO	ISO				97	3	0
Contraction	WT & KO	A6 & ISO	64	21	100	100	0	10
AVERAGE			**59**	**20**	**100**	**99**	**6**	**6**

B. ARs per myocyte by ligand binding

			Average ARs per Myocyte (% Total ARs)				
Myocyte Assay	**Genotype**	**Agonist**	**α1A**	**α1B**	**β1**	**β2**	**β3**
Binding	WT	na	**8,000 (10%)**	**21,000 (25%)**	**55,000 (65%)**	**nd**	**nd**

na=not applicable; nd=none detected

C. Myocyte phenotypes defined by ARs present

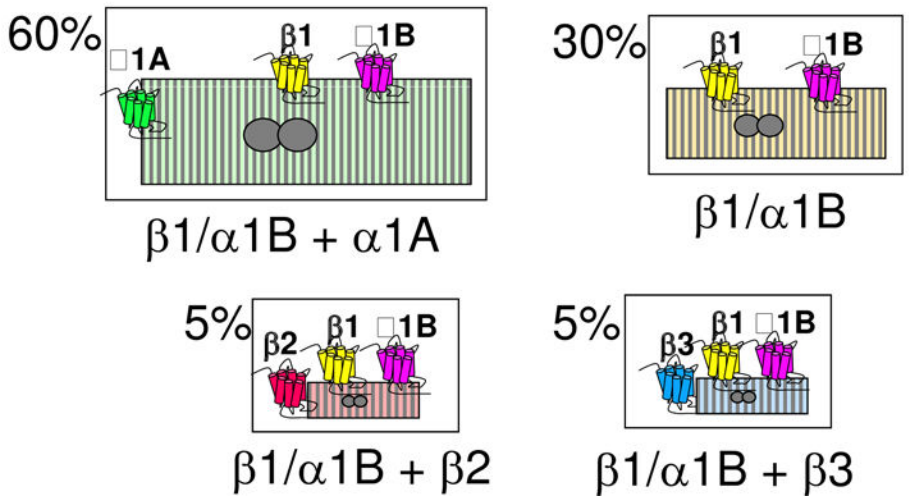


Figure 9. Summary and model of AR phenotypes of ventricular myocytes

A. Percent myocytes positive for each assay, summarized from Figures 2–8. **B**. Radioligand binding was on isolated myocytes, as in Figures 6; α1A levels are calculated to reflect that only 60% of cells have the α1A; values are rounded, and original data are in Online Figure IX. **C**. Rather than all ARs on each cell, 4 myocyte phenotypes are defined by the ARs present. The β1 and α1B are in all four phenotypes, and are the only ARs present in 30%; the α1A is also present in 60% of cells; and the β2 and β3 are each in ~5% of cells. The

α1A, β2, and β3 are largely in different cells (Figure 6). Percents are rounded from the data in panel A.