Altering the Receptor−Effector Ratio by Transgenic Overexpression of Type V Adenylyl Cyclase: Enhanced Basal Catalytic Activity and Function without Increased Cardiomyocyte β-Adrenergic Signalling[†]

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ABSTRACT: The limiting element in β-adrenergic receptor (βAR)−G_s−adenylyl cyclase (AC) signal transduction in the cardiomyocyte is not known, but it has been proposed that the level of adenylyl cyclase expression constrains βAR signaling. To alter the above equilibrium, type V AC was overexpressed in a myocyte-specific manner in the hearts of transgenic mice using the α-myosin heavy chain promoter. Expression of type V AC was ∼75% over endogenous levels as quantitated by [^{3}H]forskolin binding. Functional activity of the transgene product was evident in cardiac membrane AC studies, where basal (45 ± 11 vs 19 ± 5 pmol min^{-1} mg^{-1}) and forskolin+Mn^{2+} (695 ± 104 vs 386 ± 34 pmol min^{-1} mg^{-1}) stimulated activities were increased compared to activities in nontransgenic (NTG) littermates. However, while isoproterenol stimulated activities were higher (74 ± 12 vs 46 ± 9.8 pmol min^{-1} mg^{-1}), the fold stimulation over basal was *not* increased in ACV overexpressors compared to NTG (line $14.3 = 2.29 \pm 0.44$-fold, line $15.1 = 1.70 \pm 0.1$-fold, NTG $= 2.62 \pm 0.18$-fold). Similarly, in whole cell patch-clamp studies, βAR-mediated opening of L-type Ca^{2+} channels was not found to be enhanced in transgenic ACV myocytes (225 ± 15 vs 216 ± 10% of basal currents). Basal and isoproterenol stimulated PKA activities were elevated in the ACV mice compared to NTG, but again the extent of stimulation over basal was not enhanced. Phosphorylated phospholamban was ∼2-fold greater in myocytes from ACV hearts compared to NTG, indicating that distal elements of the contractile cascade are activated by AC overexpression. ACV mice displayed increased heart rates and fractional shortening as assessed by echocardiography. However, in vivo hemodynamic studies revealed that heart rate and contractility responses to agonist infusion were not enhanced in ACV mice compared to NTG. We conclude that at native stoichiometries, the levels of adenylyl cyclase influence basal activities and cardiac function, but do not constrain βAR signaling in the cardiomyocyte.

Like all G protein coupled receptors, the β_1- and β_2-adrenergic receptors (β_1AR, β_2AR)[1] carry out their signal transduction via coupling to guanine nucleotide binding proteins (G proteins), which subsequently activate effectors such as adenylyl cyclase. Classically, this system is considered one of amplification, in that nanomolar concentrations of agonist activating less than a full complement of receptors on the cell surface affect significant increases in intracellular second messenger (cAMP) levels. Nevertheless, the relative abundance of functional receptor, G protein, or adenylyl cyclase has the potential to ultimately define the sensitivity or efficacy of such signal transduction. For example, in most cells, βAR phosphorylation by the βAR kinase results in a decrease in agonist-promoted signaling (*1*), which is one mechanism of homologous desensitization. Such impairment of receptor−G_s coupling is functionally equivalent to a decrease in the number of receptors and suggests in cells where the phenomenon is observed that the amount of receptor may be the factor that limits signaling. Indeed, in many cell types, increasing β_2AR expression results in higher basal and agonist stimulated adenylyl cyclase activities (*2*−*4*), implying that there is sufficient G_s and adenylyl cyclase expression to accommodate increased receptor. Other studies suggest that the abundance of $G_{\alpha s}$ (*5*) or adenylyl cyclase (*6*, *7*) may be critical to the ultimate level of βAR signaling.

Most of these studies, though, have not been carried out in cell types of biological significance. Given the ∼100-fold variation in β_2AR density from different cell types in the body (*8*), two $G_{\alpha s}$ isoforms (*9*), the large number of $\beta\gamma$ subunits (*10*), and the multiple adenylyl cyclase isoforms (*11*), it is not clear which elements of the βAR−G_s−AC

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[1] Abbreviations: βAR, β-adrenergic receptor; ^{125}I-CYP, ^{125}I-cyanopindolol; αMHC, α-myosin heavy chain; I_{Ca}, L-type Ca^{2+} current density; dP/dt_{max}, first derivative of developed maximal ventricular pressure; ACV mice, transgenic mice overexpressing type V adenylyl cyclase in the heart.

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pathway are limiting factor(s) in signal transduction in various cell types. This is particularly important in cells such as the cardiomyocyte where alterations in receptor, G protein receptor kinases, G proteins, and adenylyl cyclases have been observed in diseased states such as heart failure (*12*−*17*). Indeed, depressed βAR responsiveness of the heart, as assessed in vivo and in vitro, is a hallmark of virtually every form of experimental heart failure and the human syndrome. Recently Gao et al. (*6*) used adenoviral infection techniques to overexpress type VI AC ~6-fold over endogenous levels in isolated rat neonatal myocytes. They found no increase in basal cAMP, but enhanced isoproterenol stimulated cAMP levels with such overexpression, and concluded that the amount of adenylyl cyclase sets a limit on βAR signaling in these cells. This same group has recently reported that ~20-fold overexpression of type VI AC in the hearts of transgenic mice resulted in a similar cellular phenotype (*7*). This study, though, does not specifically address whether the dominant AC isoform of the cardiomyocyte, which is type V (*18*), is a limiting factor in βAR signaling. Furthermore, the lack of an increase in basal cardiomyocyte cAMP levels in the above studies is contrary to what would be expected from such a substantial overexpression of adenylyl cyclase.

To address these issues, we have overexpressed type V AC in the hearts of transgenic mice and assessed the consequences of such on multiple biochemical aspects of signaling at base line and in response to βAR activation. By altering the equilibrium between receptor, G_s, and adenylyl cyclase, we delineated whether expression of the latter constrains βAR signaling in this relevant target cell type. And, since this was accomplished via myocyte-targeted transgenesis, we could also correlate biochemical alterations in βAR signaling to cardiac function.

EXPERIMENTAL PROCEDURES

Transgenic Mice. Cardiac-specific expression of adenylyl cyclase type V was achieved using the murine α-myosin heavy chain (αMHC) promoter that directs expression to the atria and ventricles of the heart (*19*). The cDNA for rat type V adenylyl cyclase was obtained from R. Premont, Duke University. A 4.5 kb *Xho*I digest of this construct consisted of 434 bp of 5′ ACV untranslated sequence, followed by 3787 bp of ACV coding region sequence and 231 bp of 3′ untranslated sequence. This fragment was ligated into the *Sal*I site of the full-length αMHC promoter construct (Figure 1). The integrity of the construct was confirmed by sequencing. DNA for microinjection was liberated from the αMHC−ACV plasmid by *Bam*HI digestion. Microinjection into the male pronuclei of FVB/N embryos was carried out by standard procedure. Pups were screened via Southern analysis using *Eco*RI-digested genomic DNA extracted from tail clips. The probe used to detect the presence of the transgene was a ^{32}P-labeled (by random priming method) 4 kb *Eco*RI fragment of the αMHC−ACV plasmid consisting of ~1.9 kb of the αMHC promoter and ~2.1 kb of the ACV cDNA. mRNA dot blots were carried out with total RNA from the indicated tissues as described (*20*) using the same probe as above. Transgenic mice (denoted ACV mice) were bred with nontransgenic littermates to develop heterozygous colonies. Second−third generation 18−24 week mice were studied.

Adenylyl Cyclase Activities and [^{125}I]CYP Binding. Ventricles were homogenized with a Polytron for 10 s in cold 5 mM Tris (pH 7.40), 2 mM EGTA buffer containing the protease inhibitors (5 μg/mL) leupeptin, PMSF, soybean trypsin inhibitor, benzamidine, and aprotinin. Homogenates were centrifuged at 500*g* for 10 min at 4 °C, and the pellet was discarded. The supernatant was centrifuged at 40000*g* for 10 min and the pellet resuspended in a buffer that provided for a final concentration in the reaction of 2 mM Tris, 4.8 mM $MgCl_2$, 0.8 mM EGTA, pH 7.40, with the aforementioned protease inhibitors. Adenylyl cyclase activities were measured essentially as previously described (*21*). The reaction (50 μL final volume) consisted of membranes (~10 μg) and 2.8 mM phosphoenolpyruvate, 0.06 mM GTP, 0.12 mM ATP, 0.1 mM cAMP, 4 units/mL myokinase, 10 units/mL pyruvate kinase, 0.1 mM ascorbic acid, and 3×10^6 dpm of [α-^{32}P]ATP. Reactions were at 37 °C and contained various concentrations of isoproterenol, 10 mM NaF, or 100 μM forskolin plus 3 mM Mn^{2+} (which maximally stimulates activity). Preliminary experiments showed that the generated cAMP was linear with incubation times from 2 to 12 min, and, except as noted, reactions were carried out for 10 min. Reactions were stopped by dilution with 1.0 mL of a 4 °C solution containing excess ATP and cAMP, and 25 000 dpm/mL [^{3}H]cAMP used for column recovery. [^{32}P]cAMP was separated by chromatography over alumina columns (*22*). For determination of total βAR density, reactions consisted of membranes (~50 μg) and 400 pM [^{125}I]CYP in the absence and presence of 1.0 μM alprenolol, used to define nonspecific binding (*4*). Binding reactions were carried out for 2 h at 25 °C and terminated by dilution and rapid filtration over GF/C (Whatman) filters.

[^{3}H]Forskolin Binding. [^{3}H]Forskolin binding was carried out by methods similar to those described by others in rat heart (*23*). Ventricles were homogenized as above in a 4 °C buffer consisting of 250 mM sucrose, 1 mM $MgCl_2$, 5 mM Tris, pH 7.40, and 5 μg/mL each of leupeptin, benzamidine, and soybean trypsin inhibitor and then centrifuged at 40000*g* for 10 min. The pellet was resuspended in buffer containing 8 mM $MgCl_2$, 50 mM Hepes, pH 7.4, and the above protease inhibitors. Reactions consisting of membranes (~250 μg of protein), 40 nM [^{3}H]forskolin, and varying concentrations of unlabeled forskolin were carried out for 1 h at 25 °C, and were terminated by dilution and filtration over GF/C filters. Maximal binding was calculated using the method of Motulsky (*24*).

PKA Activity. PKA activity was assessed essentially as previously described (*25*). Isolated myocytes were treated with vehicle or 10 μM isoproterenol for 5 min. Cytosolic fractions were incubated in a reaction mixture containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, 0.25 mg/mL BSA, and 50 μM Kemptide for 10 min at 30 °C. Other reactions included cAMP, thus providing for maximal stimulatable PKA activity. The reactions were stopped by spotting the assay mixture onto P81 phosphocellulose paper. The filters were washed 2 times with 1% phosphoric acid and twice with water. Bound radioactivity was measured by liquid scintillation counting.

Patch-Clamp Studies. Single ventricular myocytes were isolated from the hearts of NTG and ACV mice, and whole cell currents were recorded using patch-clamp techniques as previously described (*26*). Briefly, the heart was perfused with Ca^{2+}-free Tyrode's solution containing collagenase type

I (Worthington; 0.5 mg/mL) and bovine serum albumin (1 mg/mL) for 30−40 min by the Langendorf method at 37 °C. At the end of the perfusion period, the heart was removed, and left ventricular tissues were sieved through 200 μm nylon mesh and centrifuged for 2 min at 1000*g*. Isolated cardiomyocytes were stored in low-Cl^-, high-K^+ medium, and all experiments were performed at 20−22 °C. The patch pipets had a resistance of 2 MΩ or less. The experimental chamber (0.2 mL) was placed on a microscope stage, and the external solution changes were made rapidly using a modified Y-tube technique (*27*). The external solution contained 2 mM $CaCl_2$, 1 mM $MgCl_2$, 135 mM tetraethylammonium chloride, 5 mM 4-aminopyridine, 10 mM glucose, and 10 mM HEPES, (pH 7.3). The pipet solution consisted of 100 mM cesium aspartate, 20 mM CsCl, 1 mM $MgCl_2$, 2 mM ATP, 0.5 mM GTP, 10 mM BAPTA, and 5 mM HEPES (pH 7.3). These external and internal solutions provided isolation of Ca^{2+} channel currents from other membrane currents such as Na^+ and K^+ channel currents and also Ca^{2+} flux through the Na^+/Ca^{2+} exchanger (*28*). Data are presented as mean ± SE of *n* number of myocytes studied, which were derived from 3−5 mice.

Western Blots. Western blots for adenylyl cyclase expression were carried out using whole heart homogenates essentially as previously described (*29*) except that proteins were fractionated by 6% SDS−PAGE. Polyclonal antiserum against ACV/VI (Santa Cruz) was used at a titer of 1:200. Homogenates from COS-7 cells transiently transfected with type V AC acted as a positive control. For expression of phosphorylated phospholamban, immunoblots were carried out on cardiac extracts prepared as described (*30*). Polyclonal antiserum raised against a phospholamban peptide phosphorylated at serine 16 (PS-16, PhosphoProtein Research) was utilized at a titer of 1:5000 as previously described (*30*). Bands were quantitated using Imagequant software.

Physiologic Studies. Invasive hemodynamic studies were carried out using a closed chest, spontaneously breathing approach as described (*20*). Mice were administered ketamine (50 μg/g) and thiobutabarbital (100 μg/g), which provides for a surgical plane of anesthesia. Briefly, mice were tracheotomized to protect the airway, and catheters were placed in the right femoral artery and vein for measurement of systemic arterial pressure and infusion of drugs, respectively. A Millar Mikrotip transducer was advanced to the left ventricle via the right carotid artery for measurement of left ventricular pressure and d*P*/d*t*. Incremental doses of dobutamine were administered over a 3 min period. Responses were recorded over the last 30 s of infusion, and mice were allowed to recover between doses. Average values for arterial blood pressures, left ventricular pressures, and heart rates were measured directly from pressure waveforms meaned over a 30 s interval. Echocardiography was performed in mice sedated with intraperitoneal Avertin (1.0 mL/g of a 2.5% solution) as described (*31*).

Statistical Analysis. For in vivo hemodynamic measurements, data were analyzed by one-factor (within) or mixed, two-factor analysis of variance (ANOVA) using SUPERANOVA software by Abacus. Differences between individual means were further analyzed using single degree-of-freedom contrasts. Results from other studies were compared by *t*-tests. *P* values <0.05 were considered significant. Except as noted, data are presented as mean ± standard errors of the indicated number of independent experiments, each performed with a different mouse.

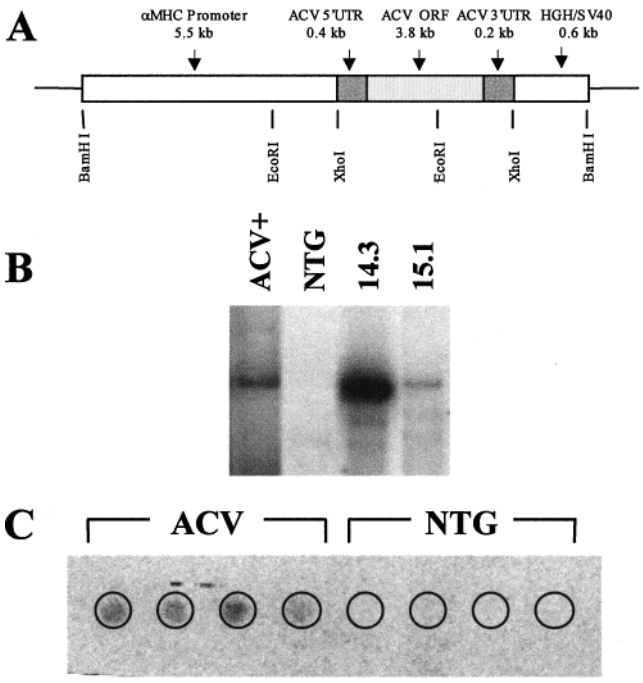

FIGURE 1: Transgenic overexpression of type V adenylyl cyclase in the heart. (A) Schematic of the construct used for transgenesis. (B) Representative Southern blot from two transgenic (TG) and one nontransgenic (NTG) mouse line. The positive control is a fragment from the rat ACV plasmid. (C) ACV mRNA overexpression in hearts from four mice of the 14.3 line.

RESULTS

Two founder mice were identified from the initial transgenesis (lines 15.1 and 14.3), and these were mated with nontransgenic littermates to produce F1−F4 generation heterozygous mice. Transgene copy number was ∼80 for the 15.1 line and >400 for the 14.3 line (Figure 1B). Litter sizes and survival of ACV transgenic mice (40 weeks of observation) were not different than those of nontransgenic mice. In transgenic mice, type V AC mRNA was increased in the heart by >10-fold (line 14.3) over nontransgenic levels (Figure 1C). Morphometric and anatomic examination of the hearts of ACV mice at 24 weeks showed no significant difference in heart to body weight ratios (6.91 ± 0.43 for the 14.3 line vs 6.24 ± 0.37 for nontransgenics, $n = 8$), and no evidence of cellular hypertrophy or fibrosis by light microscopy. Furthermore, there was no increase in mRNA levels for atrial naturetic factor, β-myosin heavy chain, or α-skeletal actin, which are sensitive indicators of hypertrophy (data not shown).

Adenylyl cyclase studies were carried out in membrane preparations from both lines in parallel with nontransgenic littermates (Figure 2). Overexpression of functional ACV transgene was confirmed by the increase in adenylyl cyclase activity evoked by forskolin+Mn^{2+}. Nontransgenic mice were found to have activities of 386 ± 34 pmol min^{-1} mg^{-1} under these conditions while the 15.1 ACV transgenic mice had activities of 695 ± 104 and the 14.3 mice had levels of 548 ± 67 pmol min^{-1} mg^{-1} ($n = 3$, $p < 0.01$ for ACV transgenics compared to nontransgenics). Basal adenylyl cyclase levels of the 15.1 and 14.3 mice were also greater

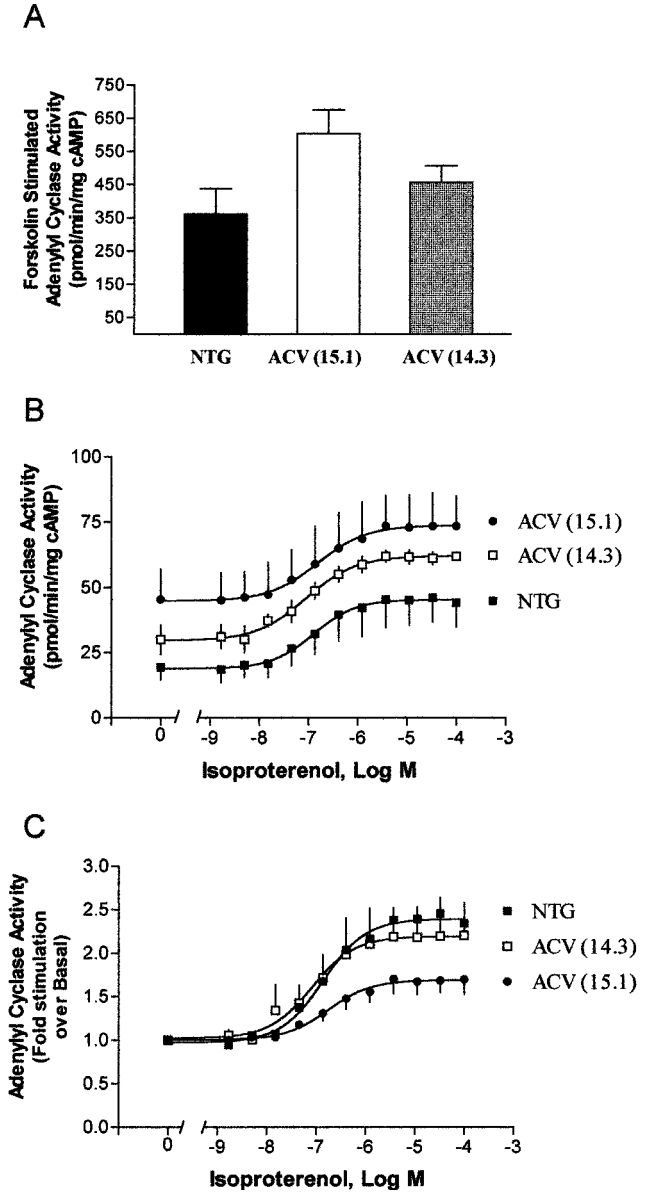

FIGURE 2: Effect of transgenic overexpression of type V adenylyl cyclase on cardiac adenylyl cyclase activities. Activities were determined in membranes as described under Experimental Procedures. In panels A and B, activities are shown in response to the indicated stimulatory agents in picomoles per minute per milligram. In panel C, agonist stimulated activities are shown as the fold stimulation over basal activities. Basal and forskolin+Mn^{2+} activities were greater in both transgenic lines compared to nontransgenic littermates ($p < 0.05$). Isoproterenol stimulated levels were also greater ($p < 0.01$), but the fold stimulation over basal levels was either not different (line 14.3) or lower (line 15.1) than nontransgenic. Results are from three experiments performed with each line.

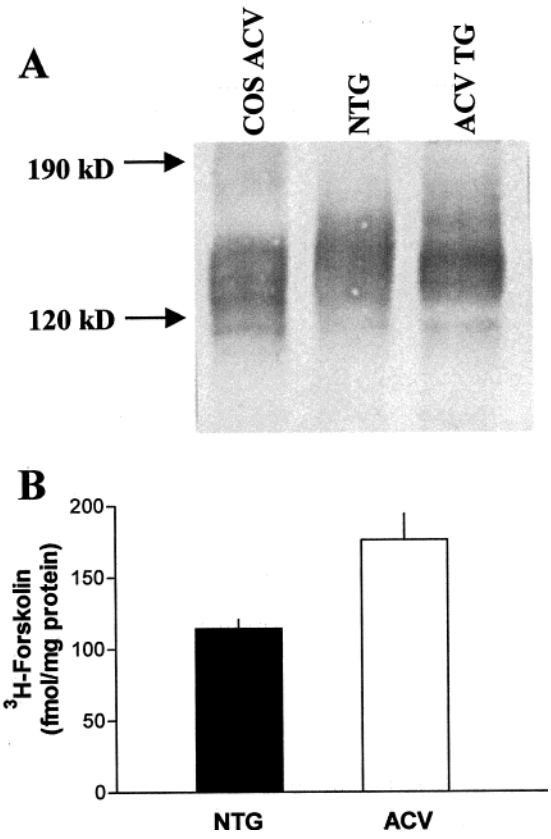

FIGURE 3: Expression of cardiac adenylyl cyclase in ACV transgenic mice. Studies were carried out in cardiac membranes as described under Experimental Procedures. (A) Results of a representative Western blot. (B) Results of [^{3}H]forskolin binding experiments showing increased (~75% over nontransgenic) binding in the transgenic mice. Results are from 5 experiments performed in triplicate.

than nontransgenic mice (45 ± 11 and 30 ± 5 vs 19 ± 5 pmol min^{-1} mg^{-1}, $n = 3$, $p < 0.05$). Isoproterenol responses are shown in Figure 2B,C. As shown, the maximal activities when expressed as picomoles per minute per milligram are greater in the transgenic mice, but this increase was of a similar magnitude as was the increase in basal activities. Indeed, when expressed as fold stimulation over basal (Figure 2C), the isoproterenol responses of the 14.3 ACV transgenic line were not different from those of nontransgenics (2.29 ± 0.44- vs 2.62 ± 0.18-fold). The isoproterenol responses of the 15.1 ACV mice (which had the higher levels of basal activities) were lower than nontransgenic (1.7 ± 0.1-fold, $p < 0.02$). Based on the reported experience with the $G_{\alpha s}$ overexpressing mice (*5*), we also assessed adenylyl cyclase activities at an early time point (2 min) after addition of agonist, forskolin, and GppNHp. The phenotype, however, remained the same, with stimulated values being increased proportionally to the increase in basal activities. The isoproterenol fold stimulation was 1.51- and 1.58-fold for ACV (line 14.3) and nontransgenic mice, respectively. Cardiac βAR expression was not different between the 15.1 and 14.3 ACV transgenic lines and nontransgenic littermates (25 ± 8.2, 20 ± 6.0 vs 21 ± 2.4 fmol/mg, $n = 3$). Subsequent studies were primarily carried out with line 14.3, which had the greatest fold stimulation of adenylyl cyclase by isoproterenol of the two transgenic lines.

Western blots revealed an increase in type V AC in the transgenic 14.3 line on the order of twice that of nontransgenic littermates (Figure 3A). This increase in ACV expression was difficult to quantitate by Western blots since the endogenous ACV signal is low. We thus utilized [^{3}H]-forskolin binding assays to quantitate the increase in protein expression as described (*23*). These studies (Figure 3B) revealed that the 14.3 ACV mice had 75% greater expression of the cyclase over that of nontransgenic littermates (175 ± 18 vs 102 ± 13 fmol/mg, $n = 5$, $p < 0.02$). It is interesting to note that the increases in basal (~33%) and forskolin+Mn^{2+} stimulated (~41%) adenylyl cyclase activities observed with this line are similar in magnitude to this increase in expression of type V adenylyl cyclase protein.

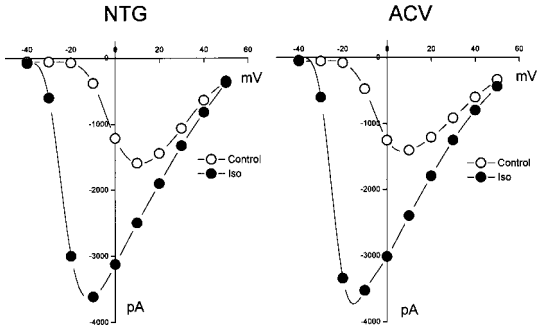

FIGURE 4: βAR signaling to L-type Ca^{2+} channels in isolated ventricular myocytes. Isoproterenol stimulated increases in Ca^{2+} currents were not different between NTG and ACV transgenic myocytes. Shown are the results from one representative set of experiments. See text for mean results from multiple experiments.

As a second approach to assessing βAR signaling that is particularly relevant to the heart, coupling of βAR to L-type Ca^{2+} channels was studied by patch-clamp experiments in isolated myocytes. Consistent with the observation that AC mice showed no change in heart to body weight or hypertrophy, the myocyte size determined by cell capacitance was normal in AC mice: the mean cell capacitance was 128.4 $\pm$ 6.2 pF ($n = 35$) and 124.7 $\pm$ 4.0 pF ($n = 55$) for ACV and NTG cells, respectively. L-type Ca^{2+} current (I_{Ca}) density in AC myocytes was also similar between the two groups: AC, 13.7 $\pm$ 0.9 pA/pF, $n = 35$ vs NTG; 13.3 $\pm$ 0.6 pA/pF, $n = 51$. Potentiation of I_{Ca} in myocytes was examined with various concentrations of Iso. Figure 4 shows typical examples of the effects of isoproterenol (1 μM) on I_{Ca} measured in NTG and ACV myocytes. Peak I_{Ca} amplitude as a function of voltage ($I-V$ relationships) before and after exposure to Iso was determined. In both groups, perfusion of isoproterenol increased the current amplitude at all test potentials measured and also shifted the mean $I-V$ relationships toward more negative potentials. Analysis of cumulative concentration−response effects of isoproterenol on peak I_{Ca} revealed the maximum increase in I_{Ca} amplitude was 216 $\pm$ 11% ($n = 17$) of basal level and 227 $\pm$ 13 ($n = 18$) for nontransgenic and ACV cells, respectively. So from these patch-clamp studies we conclude that βAR-mediated L-type Ca^{2+} channel activation was not affected by ACV overexpression.

We also assessed cellular PKA activity at base line and in response to isoproterenol exposure in isolated myocytes. As shown in Figure 5, basal PKA activities were higher in transgenic myocytes compared to nontransgenic. Isoproterenol-stimulated activities were also higher in the transgenics, but the percent increase over basal levels was the same as nontransgenic. A critical target for PKA phosphorylation in the myocyte which directly alters contractility is phospholamban (*32*). Using quantitative immunoblotting, a 2-fold increase in phosphorylated phospholamban at base line was found in ACV transgenics compared to nontransgenic littermates (Figure 6). Isoproterenol stimulated levels of phosphorylated phospholamban were similar in myocytes from both groups.

Echocardiograms were utilized for initial screening of cardiac function. In these studies ($n = 7-8$), resting heart rates (528 $\pm$ 30 vs 429 $\pm$ 25 bpm, $p = 0.027$) and fractional shortening (61 $\pm$ 2 vs 51 $\pm$ 2%, $p = 0.004$) were both higher in the ACV mice compared to nontransgenic littermates (Figure 7). Calculated left ventricular mass did not differ between

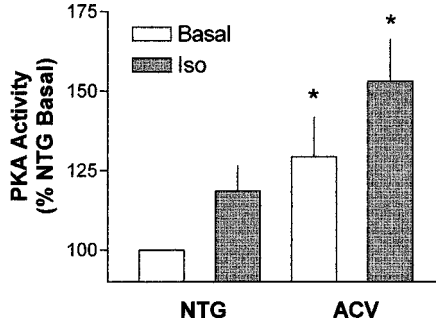

FIGURE 5: Effect of transgenic overexpression of ACV on basal and isoproterenol stimulated cardiac PKA activity. Isolated myocytes were treated with vehicle or 10 μM isoproterenol for 5 min, and PKA activity was determined from cytosolic extracts as described under Experimental Procedures. Shown are results from three experiments. (*) $p < 0.05$ vs nontransgenic values.

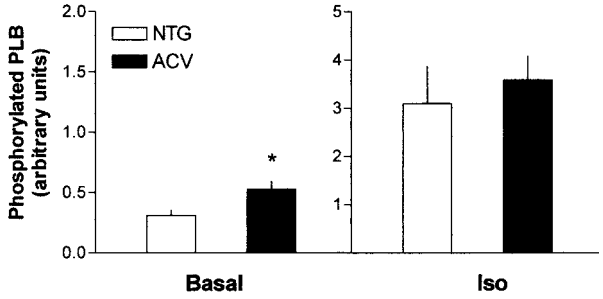

FIGURE 6: Phosphorylation of phospholamban is increased in ACV transgenic mice. Isolated myocytes were treated with vehicle or 10 μM isoproterenol for 5 min, homogenates were prepared, and 20 μg of each homogenate was electrophoresed through 4% polyacrylamide gels. Gels were transferred to 0.2 μM nitrocellulose membranes. Blots were then washed and probed with a phosphoserine-16 phospholamban polyclonal antibody. Results are from 3 experiments. (*) $p < 0.05$ vs nontransgenic values.

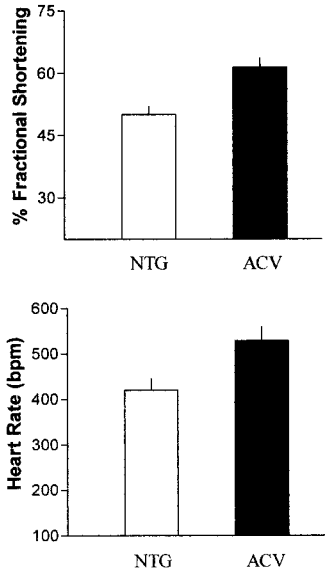

FIGURE 7: Cardiac function of ACV transgenic mice. Echocardiography was performed as described under Experimental Procedures. Results are from 7 to 8 experiments. Both heart rates ($p = 0.027$) and fractional shortening ($p = 0.004$) were greater with the ACV transgenic mice.

the two lines (data not shown). Invasive hemodynamic studies (Figure 8A,B) were undertaken to assess cardiac function at base line and in response to graded infusions of isoproterenol in the ACV overexpressing transgenic mice and

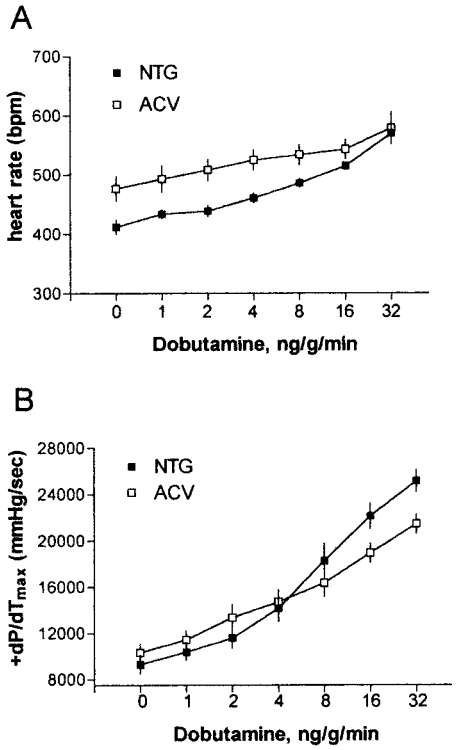

FIGURE 8: In vivo cardiac responses of ACV transgenic and nontransgenic mice. Intact mice were studied invasively as described under Experimental Procedures. In panel A, heart rates at base line and in response to infusion of dobutamine are shown. Base line rates were greater in the ACV mice, but the responsiveness to agonist was not different than that of nontransgenic mice. In panel B, contractile responses are shown, where basal + dP/dt_{max} was not different and the response to agonist was depressed in ACV mice. See text for details.

nontransgenic littermates ($n = 5$ each). Base line heart rates under these conditions were higher in ACV mice (477 ± 21 vs 412 ± 11 bpm, $p < 0.01$). Of note, this increase in basal heart rates does not appear to be due to increased βAR signaling by endogenous catecholamines, since this difference between ACV and nontransgenic mice persisted after intravenous propranolol infusion (509 ± 26 vs 445 ± 8 bpm, $p < 0.01$). The maximal heart rate response to agonist was the same between the two lines (579 ± 27 vs 570 ± 14 bpm), and the overall response as assessed by ANOVA was not different between ACV transgenics and nontransgenics. Base line + dP/dt_{max} for ACV mice was not statistically elevated compared to nontransgenic mice (10 335 ± 748 vs 9300 ± 794 mmHg/s). The +dP/dt_{max} response to isoproterenol infusion was slightly *less* in the ACV mice ($p < 0.01$ by ANOVA, Figure 8B). Taken together, the above biochemical and functional studies show no evidence of enhanced *agonist-stimulated* cardiac βAR signaling in the ACV mice, but do reveal an increase in basal adenylyl cyclase activity and cardiac function that is independent of βAR activation.

DISCUSSION

These studies were undertaken to begin to understand the limiting factors in the βAR signaling pathway of the cardiomyocyte. Type V adenylyl cyclase was overexpressed ~75% in the hearts of transgenic mice, and the biochemical and physiologic consequences were determined. We chose to overexpress type V AC because this isoform is the dominant adenylyl cyclase normally expressed in the myocyte (*18*) and we would therefore be able to clearly determine whether this element of the signal transduction cascade limits βAR function. Using four different biochemical indices, adenylyl cyclase activities, L-type Ca^{2+} channel activation, PKA activation, and phospholamban phosphorylation, we were unable to show enhanced agonist signaling by βAR in these mice. The ACV transgene was indeed functional, as forskolin+Mn^{2+} stimulated adenylyl cyclase activities were increased in the transgenic mice. While βAR signaling was not enhanced by ACV overexpression, basal adenylyl cyclase and PKA activities and phospholamban phosphorylation were clearly increased. Physiologic studies revealed a lack of agonist-promoted enhancement of cardiac inotropic, lusitropic, and chronotropic function. Base line heart rates and fractional shortening were found to be increased by echocardiography, consistent with elevated basal biochemical measurements. Thus, these results indicate that basal activities and cardiac function are set by the levels of type V adenylyl cyclase, but that adenylyl cyclase expression does not limit βAR signaling in the cardiomyocyte.

As introduced earlier, these results appear to be in contrast to those of recent reports where type VI adenylyl cyclase was overexpressed in rat neonatal myocytes by adenoviral transfection (*6*) or in myocytes by transgenesis (*7*). These investigators found no increase in basal cAMP levels but increased isoproterenol-stimulated levels in these cells, and concluded that the level of adenylyl cyclase expression is the critical limiting factor in βAR signaling in the normal heart. Furthermore, resting cardiac function was not altered, but β-agonist stimulated parameters were increased. There are, however, several differences in the two transgenic studies including the apparent much greater extent of overexpression in the aforementioned study. Also, we overexpressed type V adenylyl cyclase, as compared to the study of Gao et al. (*7*) where type VI was overexpressed. The two isoforms are generally considered similar in regard to function and regulation, but some differences have been identified. Types V and VI adenylyl cyclases constitute the "type V" family of the adenylyl cyclases (*9*, *33*), and share approximately 75% amino acid homology. Both isoforms are activated by G_s and inhibited by G_i. ACV and VI, in contrast to some other AC isoforms, are not regulated by Ca^{2+}/calmodulin. Both, however, are inhibited by submicromolar concentrations of Ca^{2+} (*34*−*36*). Regarding this latter regulation, it is interesting to consider that the functional consequences of type V or VI adenylyl cyclase overexpression may be negatively modulated in the intact myocyte by enhanced cAMP/PKA-dependent calcium influx. This cannot be the basis of our findings of unaltered βAR stimulation over basal activities, since these adenylyl cyclase assays were performed with washed membranes, and we failed to observe altered Ca^{2+} channel activity. In contrast to the above similarities between the two adenylyl cyclases, regulation of these two adenylyl cyclases by protein kinase C appears to be isoform-specific. Recent work by Lai et al. (*37*) demonstrated that the ACVI isoform is directly phosphorylated and inhibited by PKCδ and PKCϵ, which are known to be expressed in the heart. ACV, alternatively, is stimulated by PKCα and PKCζ (*38*). It appears that PKA phosphorylates and inhibits both ACV and ACVI (*39*, *40*). By sequence analysis, though, ACV has one putative PKA phosphorylation site, whereas the ACVI isoform has two such sites.

In the current work we utilized transgenesis to moderately overexpress adenylyl cyclase. This absolute level of change is typical of what occurs in some models of heart failure where levels are decreased (*17, 41*) or the levels of increase that can realistically be expected from gene therapy. In contrast, in the studies by Gao et al., type VI was overexpressed ~6-fold using a transient adenoviral infection technique (*6*) and ~20-fold by transgenesis (*7*). The subcellular distribution of adenylyl cyclase is more likely to be different than endogenous distribution with such extensive overexpression, and thus there may have been the potential for promiscuous coupling in these studies. Finally, these type VI studies utilized cAMP measurements as the indicator of in vitro receptor function, while we examined adenylyl cyclase activities, L-type Ca^{2+} channel activity, and PKA activity. Like our in vitro findings, the ACV mice were found to be identical physiologically to nontransgenic mice in their *response to β-agonist*, consistent with our hypothesis that agonist-promoted βAR function is not limited by adenylyl cyclase expression. It would not be unexpected, though, that base line cardiac function (such as heart rate or contractility) would be increased to some extent in the ACV mice, since basal adenylyl cyclase activities, PKA activities, and phospholamban phosphorylation are in fact increased. This was indeed observed in the echocardiographic studies where resting heart rate and fractional shortening were elevated in the transgenic mice. In the invasive studies, resting contractility was not significantly elevated. However, given the greater variability of these measurements performed after surgical manipulation, it is unlikely that a 10-percentile point increase in fractional shortening could be detected by d*P*/d*t* measurements. Resting heart rates, though, were increased in the transgenics. This was not due to endogenous catecholamine activation of the receptor, since propranolol administration (using a previously established dose that blocks infused agonist stimulated heart rates) failed to equalize heart rates between the two lines. Interestingly, the absolute maximal heart rate and contractility (Figure 8) of the ACV transgenics in response to agonist were not different than nontransgenic even though the absolute adenylyl cyclase levels (Figure 2) were increased. This is consistent, though, with the phospholamban results, which showed a similar level of isoproterenol stimulated phosphorylation despite the higher levels of adenylyl cyclase and PKA activities. This suggests that phospholamban may have a role in limiting the physiologic response.

We have previously shown that moderate overexpression of the β_2AR in transgenic mice raises basal levels of adenylyl cyclase as well as isoproterenol stimulated levels, in terms of both absolute levels and the fold stimulation over basal (*4*). It is thus suggested, based on these results, that βAR expression is limiting in the myocyte, and, as a corollary, the amounts of G_s and adenylyl cyclase present are sufficient to accommodate, to some extent, increased receptor expression. Of note, the level of overexpression of the β_2AR in our previous study (*4*) was ~45-fold over background. Yet, the increases in basal and isoproterenol stimulated activities were on the order of 3- and 5-fold over nontransgenics, respectively. Likely, then, in those studies we did exceed the capacity of one or more signal transduction components downstream of the receptor since the increase in stimulation was not comparable to the increase in receptor expression. Transgenic overexpression (~2.5-fold) of $G_{\alpha s}$ in the heart has also been reported (*5*). Basal, isoproterenol, and NaF stimulated adenylyl cyclase activities were minimally increased in cardiac membranes from these mice, and the percentage of βAR in the high-affinity state was noted to be increased. This would suggest the level of $G_{\alpha s}$ is not the major component that limits βAR signaling in the heart. Interestingly, the $G_{\alpha s}$ mice have been reported to have enhanced L-type Ca^{2+} activity through a non-cAMP-dependent mechanism (*42*). And, these mice develop a cardiomyopathy in senescence, indicating that such overexpression (and by inference acquired signaling) has pathologic significance (*43*). Nevertheless, evidence for significantly enhanced receptor coupling to adenylyl cyclase is not compelling in these mice.

Our results from the current study, that adenylyl cyclase is not the limiting factor in βAR signaling in the heart, are relevant within the context of the normal heart. Thus, processes that ultimately lead to a decrease in adenylyl cyclase expression might transition the equilibrium between receptor, G_s, and adenylyl cyclase such that the latter would serve to constrain βAR signaling. Interestingly, several models of cardiac failure are associated with decreases in adenylyl cyclase mRNA levels (*17, 41*) or forskolin stimulated activities (*17, 41, 44*). Other mechanisms of βAR dysfunction often coexist in models of hypertrophy or failure, including decreases in βAR expression, receptor coupling to G_s, and increases in G_i, so it is not clear what the impact of restoring adenylyl cyclase levels to normal levels in these models would be.

In conclusion, we have investigated the relationship between βAR and the effector adenylyl cyclase in the heart by transgenically overexpressing the type V isoform. Such overexpression enhanced catalytic activity under basal conditions and in response to forskolin. However, βAR signaling, assessed as adenylyl cyclase activity, L-type Ca^{2+} channel opening, and PKA activity, was not enhanced by such overexpression. Consistent with these findings, base line heart rate and contractility were increased, but the in vivo response to agonist was not enhanced. Thus, in the normal myocyte the relative levels of the components of the βAR−G_s−AC transduction pathway are such that βAR signaling is not limited by expression of adenylyl cyclase. In contrast, basal activities and cardiac function can be affected by increases (and presumably decreases) in the level of type V adenylyl cyclase expression.

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