Type 5 Adenylyl Cyclase Disruption Alters Not Only Sympathetic But Also Parasympathetic and Calcium-Mediated Cardiac Regulation

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Abstract—In a genetically engineered mouse line with disruption of type 5 adenylyl cyclase ($AC5^{-/-}$), a major cardiac isoform, there was no compensatory increase in other isoforms of AC in the heart. Both basal and isoproterenol (ISO)-stimulated AC activities were decreased by 30% to 40% in cardiac membranes. The reduced AC activity did not affect cardiac function (left ventricular ejection fraction [LVEF]) at baseline. However, increases in LVEF after ISO were significantly attenuated in $AC5^{-/-}$ ($P<0.05$, n=11). Paradoxically, conscious $AC5^{-/-}$ mice had a higher heart rate compared with wild-type (WT) mice (613±8 versus 523±11 bpm, $P<0.01$, n=14 to 15). Muscarinic agonists decreased AC activity, LVEF, and heart rate more in WT than in $AC5^{-/-}$. In addition, baroreflex-mediated, ie, neuronally regulated, bradycardia after phenylephrine was also attenuated in $AC5^{-/-}$. The carbachol-activated outward potassium current (at −40 mV) normalized to cell capacitance in $AC5^{-/-}$ (2.6±0.4 pA/pF, n=16) was similar to WT (2.9±0.3 pA/pF, n=27), but calcium (Ca^{2+})-mediated inhibition of AC activity and Ca^{2+} channel function were diminished in $AC5^{-/-}$. Thus, $AC5^{-/-}$ attenuates sympathetic responsiveness and also impairs parasympathetic and Ca^{2+}-mediated regulation of the heart, indicating that those actions are not only regulated at the level of the receptor and G-protein but also at the level of type 5 AC. **(*Circ Res*. 2003;93:364-371.)**

Key Words: β-adrenergic receptors ■ muscarinic receptors ■ calcium channels ■ knockout ■ adenylyl cyclase isoforms

Cardiac rate and contractility are regulated by both sympathetic and parasympathetic mechanisms. Sympathetic stimulation leads to coupling of the β-adrenergic receptor (β-AR) and Gs, the G-protein responsible for stimulating activity of adenylyl cyclase (AC), a membrane-bound enzyme that catalyzes the conversion of ATP to cAMP, thereby stimulating protein kinase A and ultimately increasing cardiac contractility.[1–4] Parasympathetic stimulation counteracts these effects through the activation of the muscarinic receptor and Gi, the G-protein that can inhibit cardiac contractility and rate.[5] These sympathetic and parasympathetic mechanisms constitute the two arms of autonomic regulation of the heart. A considerable amount of data exists relating to autonomic regulation at the level of their respective receptors and G-proteins. A novel concept to consider, central to this study, is whether parasympathetic regulation also occurs at the level of AC.

The goal of the present investigation was to determine the regulation of cardiac contraction and rate by type 5 AC in response to β-AR stimulation and also whether it can modulate parasympathetic function in vivo. Whereas all prior studies have examined these questions in vitro[6] or in vivo[7,8] using pharmacological stimulation or even by overexpressing isoforms of AC in the heart,[9–11] we selected the approach of targeted disruption of AC. However, this experimental design is complicated by the fact that AC consists of 9 mammalian transmembrane isoforms.[4,12–14] We selected type 5 AC to knockout in the mouse ($AC5^{-/-}$), because this isoform is one of the most prominent in adult cardiac tissue and is expressed negligibly in other organs except for the brain.[4,15,16] Furthermore, whereas all of the 9 AC isoforms so far isolated can be linked to Gs stimulation, Gi inhibition is associated with only a few AC isoforms, eg, types 1, 5, and 6 AC, and has been observed only in vitro.[17,18] In addition, type 5 AC also is inhibited directly by low concentrations of calcium (Ca^{2+}).[19] Therefore, we also examined the regulation of AC activity by Ca^{2+}.[20] The specific questions we addressed in this study are whether elimination of type 5 AC decreases either baseline cardiac function or heart rate (HR), impairs sympathetic stimulation, or alters parasympathetic modulation of cardiac function and HR. We addressed these questions using a combination of in vivo and in vitro approaches, eg, by measuring cardiac function echocardiographically, measuring

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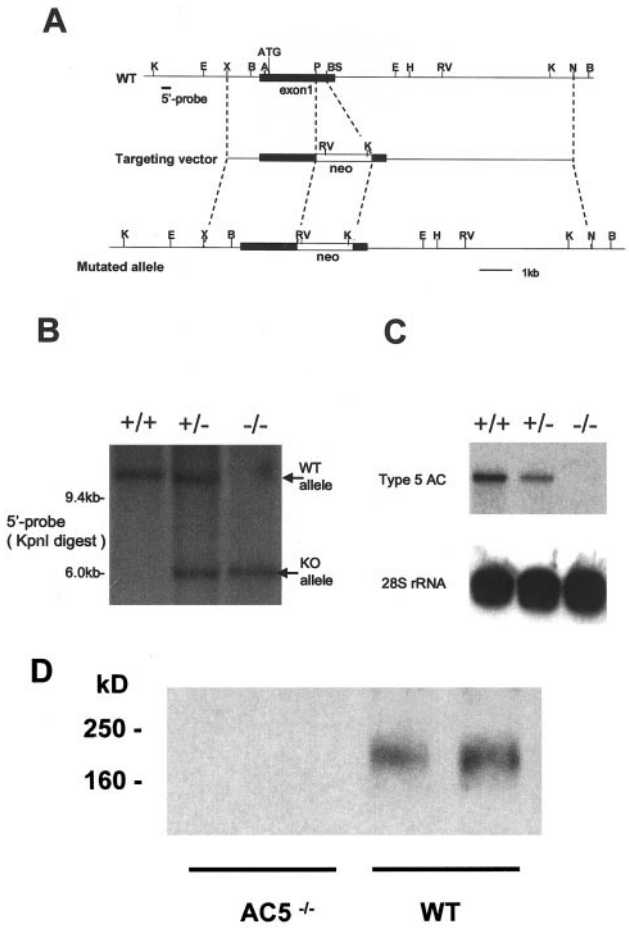

Figure 1. Generation of *AC5*$^{-/-}$. A, Targeted disruption of the type 5 AC gene. Partial structure of the type 5 AC gene (WT), targeting vector construct (targeting vector), and resultant mutated allele are shown. The positions of the phosphoglycerate kinase promoter neo cassette (neo) and 5′ probe (400 bp) are indicated. K indicates *Kpn*I; E, *Eco*RI; X, *Xho*I; A, *Apa*I; P, *Pst*I; BS, *Bss*HII; H, *Hind*III; RV, *Eco*RV; N, *Nco*I; and B, *Bam*HI. B, Southern blot analysis of genomic DNA from the offspring of F1 heterozygote intercross. C, RNase protection assay of type 5 AC and 28S rRNA in the heart of WT (+/+), heterozygous (+/−), and homozygous (−/−) mice. D, Western blot analyses of *AC5*$^{-/-}$ mouse heart compared with WT using type 5 AC–specific antibody.

HR in conscious mice, measuring Ca^{2+} channel activity of isolated myocytes, and assessing AC activity in vitro in cardiac membranes.

Materials and Methods

Generation of Knockout Mice

All mice were 129/SvJ-C57BL/6 mixed-background littermates from F1 heterozygote crosses. All experiments were performed in 4- to 6-month-old homozygous *AC5*$^{-/-}$ and wild-type (WT) littermates. This study was approved by the Animal Care and Use Committee at New Jersey Medical School (see the online supplement, available at http://www.circresaha.org, for additional detail).

RNase Protection Assay

Partial fragments of mouse AC cDNA clones for each isoform (types 1 through 9) were obtained by polymerase chain reaction. A human 28S ribosomal RNA probe was used as an internal control. RNase protection assay was performed using the RPA III kit (Ambion) as suggested by the manufacturer.

AC Assay

Hearts were dissected from the mice, and membrane preparations were prepared as described previously.[21] For the study of Ca^{2+} inhibition, the membranes were treated first with EGTA to extract the endogenous Ca^{2+} before the assay. Free Ca^{2+} concentrations were obtained with the use of 200-μmol/L EGTA buffers, as described previously.[22,23]

Physiological Studies

ECG wires, a jugular vein catheter for drug infusion, and a femoral artery catheter for arterial pressure monitoring were implanted under anesthesia as described previously.[24,25] Measurements of left ventricular ejection fraction (LVEF) were taken using echocardiography under anesthesia with 2.5% tribromoethanol (0.010 to 0.015 mL per gram of body weight) injected intraperitoneally.[26,27] Intravenous infusion of ISO (0.04 μg/kg per min IV for 5 minutes) was performed using an infusion pump. To examine the responses to a muscarinic agonist, acetylcholine (ACh) (25 mg/kg IP) was coadministered intraperitoneally during the intravenous infusion of ISO (0.04 μg/kg per min). In addition, in conscious mice, ACh (0.01 and 0.05 mg/kg), atropine (0.25, 1, and 2 mg/kg), or verapamil (0.75 mg/kg) was administered intravenously, and the ECG was recorded. A recovery period of 15 minutes was allowed for the HR to return to baseline before administering the next drug. To examine HR responses to baroreflex hypertension, phenylephrine (0.2 mg/kg IV) was infused and the ECG and arterial pressure were measured.

Pathology

The pathological examination included assessment of body weight, heart weight, and light microscopy of H&E-stained sections of the left ventricle.

Radioligand Binding Assays and Western Blotting

Radioligand binding assays for β-AR were conducted using the above membrane preparations and ^{125}I-cyanopindolol as previously described.[28] Western blotting was conducted using commercially available antibodies, except for type 5 AC (see the online data supplement).

Electrophysiological Studies

Whole-cell currents were recorded using patch-clamp techniques as previously described.[29–32] (See the online data supplement for additional detail.)

Statistical Analysis

All data are reported as mean±SEM. Comparisons between *AC5*$^{-/-}$ and WT values were made using a Student's *t* test. For statistical analysis of data from multiple groups, one-way ANOVA was used with Bonferroni post hoc test. $P<0.05$ was taken as a minimal level of significance.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Targeted Disruption of the Type 5 AC Gene

The type 5 AC gene was disrupted in mice using homologous recombination (Figure 1A). Mice were genotyped by Southern blotting using genomic DNA from tail biopsies (Figure 1B). mRNA expression of type 5 AC was undetectable in *AC5*$^{-/-}$ (Figure 1C). Type 5 AC cardiac protein was undetectable in *AC5*$^{-/-}$ (Figure 1D). The growth, general appearance, and heart size of the *AC5*$^{-/-}$ were similar to WT (Table). Normal cardiac architecture was demonstrated on light microscopy (see the online data supplement).

Heart Size and Cardiac Function

	WT (n)	$AC5^{-/-}$ (n)
Age, mo	4.4±0.1 (15)	4.2±0.2 (14)
BW, g	25±1 (15)	27±1 (14)
LV/BW, mg/g	3.9±0.1 (9)	4.1±0.2 (8)
HR, bpm	523±11 (15)	613±8 (14)*
LVDD, mm	3.9±0.1 (11)	4.0±0.1 (10)
LVSD, mm	2.6±0.09 (11)	2.7±0.1 (10)
LVEF, %	70±1.2 (11)	70±1.5 (10)
%FS	33±0.9 (11)	33±1.0 (10)

Data are mean±SEM. HR is under conscious state and other functional data are under anesthesia. LVEF indicates left ventricular ejection fraction; LVDD, LV end-diastolic diameter; LVSD, LV end-systolic diameter; and %FS, % fractional shortening.

*$P<0.01$.

No Compensatory Increase in the Other Isoforms of AC

We then examined whether there were compensatory increases in the expression of the other isoforms of AC in $AC5^{-/-}$. Because AC isoform–specific antibodies that can convincingly determine the level of protein expression of all the isoforms are not available, we quantitated the mRNA expression of the AC isoforms by RNase protection assays. cRNA of the 28S rRNA was used as an internal control. Type 2, 3, 4, 6, 7, and 9 AC were detected readily but not increased (Figure 2), whereas types 1 and 8 were hardly detectable (data not shown), indicating that type 6 AC, a homologue of type 5 AC in the heart, could not compensate for the type 5 AC deficiency.

AC Activity Was Decreased in the Heart of $AC5^{-/-}$ In Vitro

AC activity was decreased in $AC5^{-/-}$ relative to that in WT by 35±4% (basal), 27±5% (ISO), 27±2% (GTPγS), and 40±5% (forskolin) (Figure 3A). More specifically, ISO increased AC activity by 78±6 pmol/15 min per mg in WT but only 64±4 pmol/15 min per mg in $AC5^{-/-}$, indicating that the response to ISO was attenuated in $AC5^{-/-}$. These data indicate that type 5 AC is responsible for ≈30% to 40% of total AC activity in the mouse heart. Carbachol (10 μmol/L), a muscarinic agonist, decreased ISO-stimulated AC activity by 21±3% in WT, but this was hardly detectable in $AC5^{-/-}$ (Figure 3B), indicating that muscarinic (Gi-induced) inhibition of the AC activity is markedly attenuated in $AC5^{-/-}$.

Regulation of AC Activity by Free Ca^{2+}

To investigate the modulation of AC activity by free Ca^{2+}, we examined cAMP production in membranes from the hearts of WT and $AC5^{-/-}$ at different Ca^{2+} concentrations in the presence of ISO (100 μmol/L ISO+100 μmol/L GTP) (Figure 3C). The ISO-stimulated AC activity was inhibited by increasing concentrations of Ca^{2+} as expected in WT. The Ca^{2+} inhibition of AC activity was impaired in $AC5^{-/-}$. The reduction in magnitude of inhibition was most apparent in $AC5^{-/-}$, ie, in the submicromolar range of Ca^{2+} (Figure 3C).

Basal Cardiac Function Was Not Decreased, but the Response to ISO and Muscarinic Inhibition of ISO Were Impaired

We originally hypothesized that cardiac function, both basal and ISO-stimulated, would be depressed in $AC5^{-/-}$. The cardiac responses to intravenous ISO on LVEF in $AC5^{-/-}$ were attenuated as expected (Figure 4). However, baseline cardiac function was not different between WT and $AC5^{-/-}$ (LVEF: WT versus $AC5^{-/-}$, 70±1.2% versus 70±1.5%, n=10 to 11; fractional shortening: WT versus $AC5^{-/-}$, 33±0.9% versus 33±1.0%, n=10 to 11) (Table). Muscarinic inhibition of ISO-stimulated cardiac function, as measured by LVEF, was prominent in WT, as expected, but was attenuated

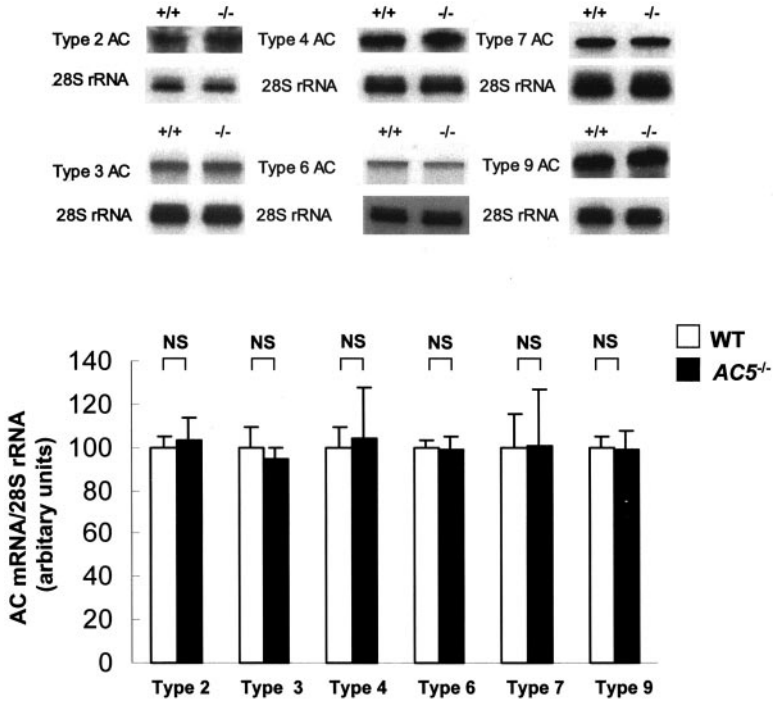

Figure 2. RNase protection assays of types 2, 3, 4, 6, 7, and 9 AC and 28S rRNA in the hearts of 4 to 6 pairs of WT (+/+) and $AC5^{-/-}$ (−/−). cRNA of the 28S rRNA was used as an internal control. Types 1 and 8 AC were hardly detectable (data not shown). Representative autoradiographs of each AC isoform and 28S rRNA are shown in the top panel. Quantitation of relative intensities of each AC isoform to 28S rRNA is shown in the bar graph. NS indicates not significant

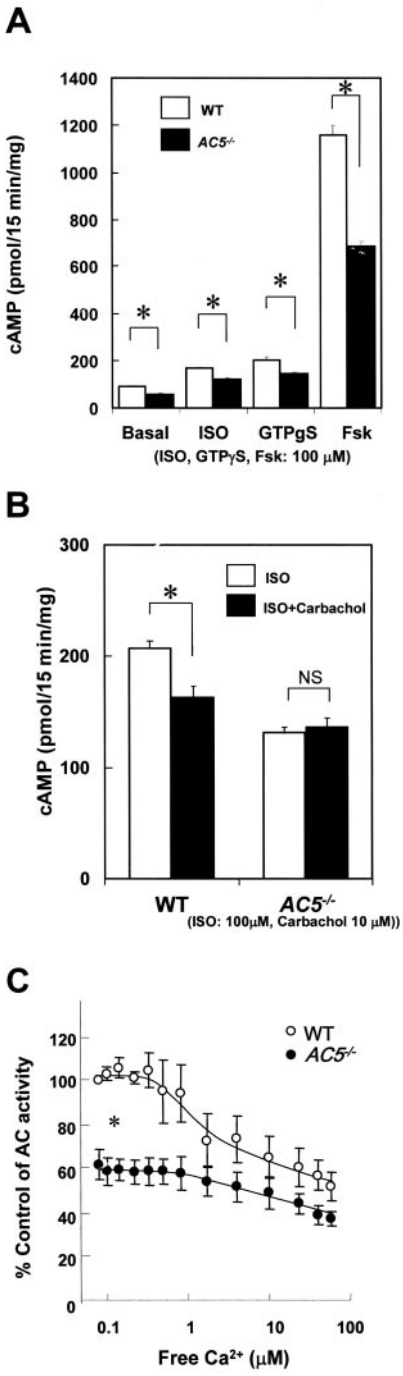

Figure 3. Adrenergic, muscarinic, and Ca^{2+}-mediated regulation of cardiac AC activity. A, AC activity in vitro. The steady-state AC activity was determined as the maximal cAMP production over 15 minutes. Stimulation was performed at the level of the β-AR (ISO), G-protein (GTPγS), and AC catalytic unit with forskolin (Fsk). *$P<0.01$, n=5. B, Effects of carbachol on AC activity. AC was preactivated by ISO. AC activity was then determined in the absence and presence of carbachol. Carbachol at 10 μmol/L produced its maximal inhibitory effect. *$P<0.01$, n=5. C, To investigate the inhibition of AC activity by Ca^{2+}, we examined cAMP production in membranes from the hearts of WT and $AC5^{-/-}$ at increasing Ca^{2+} concentrations in the presence of ISO. ISO-stimulated AC activity was inhibited more in WT than in $AC5^{-/-}$. The value at 0.08 μmol/L free Ca^{2+} was taken as 100%. *$P<0.05$ using ANOVA for all data points except 1.7 μmol/L.

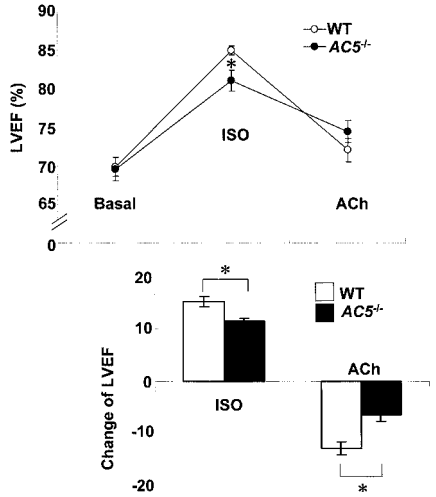

Figure 4. Response of cardiac function to ISO or ACh in vivo. LVEF in response to β-AR stimulation with ISO, 0.04 μg/kg per min IV, was significantly attenuated in $AC5^{-/-}$. ACh superimposed on ISO reduced LVEF in WT, but its inhibition was attenuated in $AC5^{-/-}$. Absolute values of the responses to ISO and of ACh in the presence of ISO are plotted at the top. Bars representing the absolute changes in responses of LVEF to ISO and then to ACh in the presence of ISO are plotted on the bottom. *$P<0.05$, n=11.

in $AC5^{-/-}$ (Figure 4), suggesting that muscarinic inhibition of β-adrenergic stimulation was impaired. This conclusion is based on the finding that ACh in the presence of ISO reduced LVEF less in $AC5^{-/-}$ than WT ($P<0.05$). However, because the baseline during ISO was lower in $AC5^{-/-}$, the level achieved after ACh was not significantly different.

Parasympathetic (Muscarinic) Control of HR

Baseline HR was significantly elevated in conscious $AC5^{-/-}$ (WT versus $AC5^{-/-}$: 523±11 versus 613±8 bpm, $P<0.01$, n=14 to 15) (Table). The increase in HR after muscarinic receptor blockade by atropine (1 mg/kg IV) in WT was not observed in $AC5^{-/-}$ (Figure 5A). Muscarinic stimulation in conscious WT with ACh (0.01 mg/kg IV) decreased HR by 15% but significantly less (1.3%) in $AC5^{-/-}$ (Figure 5B). However, high doses of ACh (0.05 mg/kg IV) decreased HR similarly in both WT and $AC5^{-/-}$. At the higher doses of ACh, it is possible that the lack of AC5 inhibition was overwhelmed. In contrast, verapamil, which decreases HR through a nonmuscarinic mechanism, reduced HR in $AC5^{-/-}$ and WT similarly (−33±10 versus −36±10 bpm). These findings suggest that muscarinic inhibition was impaired in the conscious state in the absence of ISO stimulation in $AC5^{-/-}$.

To confirm that muscarinic, and therefore parasympathetic, neural regulation of the heart was changed, we injected phenylephrine (0.2 mg/kg IV) to elevate arterial pressure transiently through vasoconstriction and to induce baroreflex-mediated slowing of HR. Phenylephrine increased systolic arterial pressure similarly in both WT and $AC5^{-/-}$. However, the degree of HR slowing was significantly less in $AC5^{-/-}$ than in WT (Figure 5C), suggesting that the baroreflex, most likely through its parasympathetic control, was attenuated in $AC5^{-/-}$.

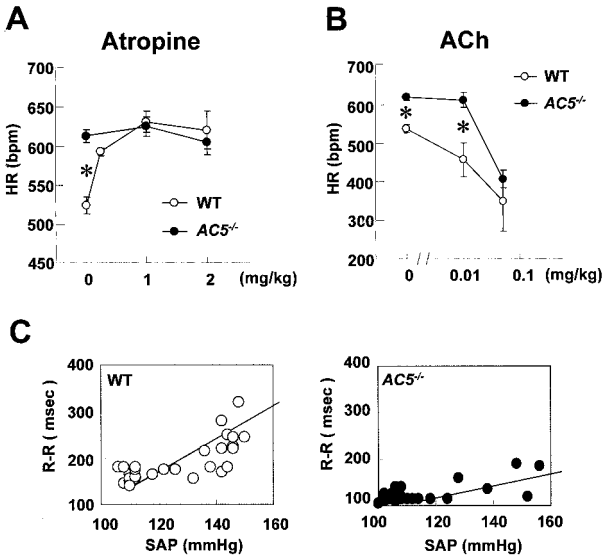

Figure 5. Muscarinic regulation of cardiac function in vivo. A, Effects of atropine. Baseline HR was significantly elevated in conscious *AC5*$^{-/-}$. **P*<0.01, n=14 to 15. Administration of atropine increased HR dose dependently in conscious WT, but the elevation by atropine was impaired in *AC5*$^{-/-}$. B, Effect of ACh. Administration of ACh attenuated HR dose dependently in conscious WT, but the inhibition by ACh at the dose of 0.01 mg/kg was impaired in *AC5*$^{-/-}$. **P*<0.01, n=5. C, Baroreflex regulation of HR. Baroreflex slowing of HR in response to phenylephrine-induced increase in arterial pressure is shown by the plot of systolic arterial pressure (SAP) versus the inverse of heart rate, ie, the R-R interval (ms). The depressed slope indicates that reflex parasympathetic bradycardia was impaired in *AC5*$^{-/-}$.

β-AR Binding Assay and Western Blotting

The expression of β-AR was not different (Kd: WT 102±17 pmol/L, *AC5*$^{-/-}$ 115±29 pmol/L; Bmax: WT 36±5 fmol/mg, *AC5*$^{-/-}$ 31±4 fmol/mg; n=5, *P*=NS), nor was the expression of Gsα, Giα, Gqα, Gβγ, β-ARK, α_1-AR, or muscarinic receptor type 2 (Figure 6A).

K$^+$ Current Activity

To determine whether enhanced baseline HR and blunted response to muscarinic agonists in *AC5*$^{-/-}$ are attributable to changes in the K$^+$ channel, we examined muscarinic receptor–coupled K$^+$ channel currents in atrial myocytes.[30,33–36] Figure 6B shows representative atrial K$^+$ channel currents induced by carbachol (10 μmol/L) recorded in WT and *AC5*$^{-/-}$ myocytes. Rapid application of carbachol elicited an outward K$^+$ current via Gi proteins. The carbachol-induced currents rose quickly to a peak and then decayed slowly to a steady level. The peak amplitude and decay time were similar between WT and *AC5*$^{-/-}$ myocytes (Figure 6C). These results indicate that coupling between muscarinic receptors and the Gi-gated K$^+$ channel is not altered in *AC5*$^{-/-}$ myocytes.

Basal Ca^{2+} Channel Activity and Response to ISO

Peak inward I_{Ca} amplitude (with 5 mmol/L EGTA in the pipette solution), normalized to cell capacitance (I_{Ca} density), was similar in myocytes isolated from *AC5*$^{-/-}$ (7.1±0.3 pA/pF, n=69) and WT (6.7±0.3 pA/pF, n=55). Half decay time of I_{Ca} at +10 mV was 21.9±1.4 and 21.0±1.4 ms for *AC5*$^{-/-}$ and WT, respectively. These data suggest that

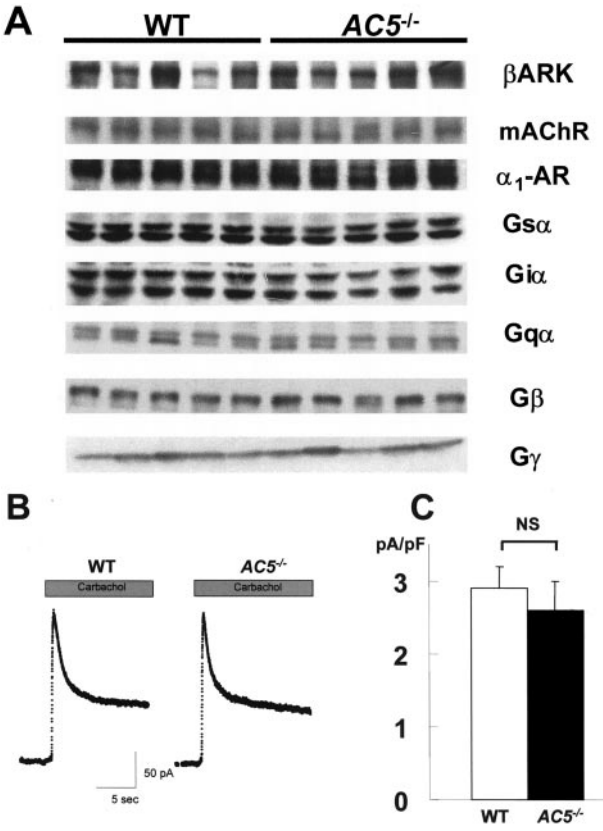

Figure 6. A, Western blot analysis for protein expression of Gsα, Giα, Gqα, Gβ, Gγ, and β-adrenergic receptor kinase (β-ARK) as well as α_1-AR and muscarinic receptor type 2 (mAChR) in WT and *AC5*$^{-/-}$. There were no differences in any of these proteins in *AC5*$^{-/-}$. B, Carbachol-activated K$^+$ current in atrial myocytes isolated from WT and *AC5*$^{-/-}$. The cells are held at −40 mV, and carbachol was applied as indicated in the bar above each trace. C, Mean carbachol-induced current density. Peak outward K$^+$ currents were normalized to cell capacitance to yield current density (pA/pF). Data are mean±SEM of WT (n=27) and *AC5*$^{-/-}$ (n=16) cells.

changes in AC activity did not directly influence Ca^{2+} channel density or inactivation kinetics. In previous studies, we have proposed that AC activity and subsequent cAMP synthesis, which modulate Ca^{2+} channel activity, are regulated by Ca^{2+} influx through the channel.[20,37] We thus compared the effects of ISO on I_{Ca} using procedures designed to modulate the cytoplasmic Ca^{2+} concentration with two different Ca^{2+} chelators, EGTA and BAPTA, the latter of which have faster Ca^{2+} binding kinetics, and with the use of extracellular barium (Ba^{2+}), which permeates the Ca^{2+} channel but does not trigger Ca^{2+} of the sarcoplasmic reticulum (SR). Figure 7A shows a typical example of the effect of ISO (1 μmol/L) on I_{Ca} in WT and *AC5*$^{-/-}$. In both groups, ISO increased the current amplitude at all test potentials and also shifted the *I*-V relationships toward more negative potentials. However, in the presence of ISO, peak I_{Ca} amplitude in *AC5*$^{-/-}$ was significantly smaller (−19.6±2.0 pA/pF, *P*<0.05). Analysis of cumulative dose-response effects of ISO (Figure 7B) revealed that, when either BAPTA or Ba^{2+} was used, the maximum response of the Ca^{2+} channel to ISO was significantly augmented (≈2.4-fold) compared with cells

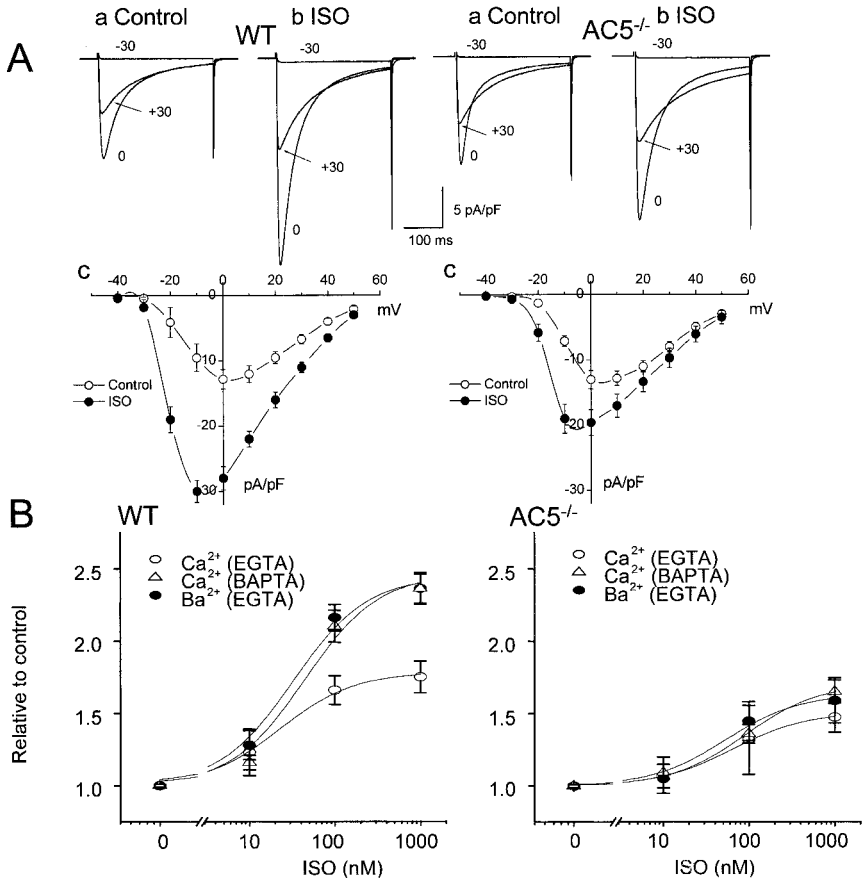

Figure 7. A, Effects of ISO on I_{Ca} in WT and *AC5*$^{-/-}$ myocytes. Traces show currents recorded from a holding potential of −50 mV to indicated potentials in control before (a) and after (b) application of ISO (1 μmol/L). In c, peak I_{Ca} was normalized to the cell capacitance to give current densities (pA/pF), which were plotted as a function of voltage. Data are mean±SEM, n=7, WT and n=4, *AC5*$^{-/-}$ myocytes. There was no difference in control I_{Ca} density between WT and *AC5*$^{-/-}$ myocytes. In the presence of ISO, peak I_{Ca} amplitude in WT and *AC5*$^{-/-}$ was significantly different (−30±1.7 versus −19.6±2.0 pA/pF, $P<0.05$). B, Concentration-dependent effects of ISO on I_{Ca} measured in myocytes dialyzed with EGTA or BAPTA and on Ba^{2+} currents with EGTA. The relative increase of peak current amplitude was plotted against ISO concentration. The solid lines were best fit to one-to-one binding model. Data are from 8 to 30 myocytes.

dialyzed with EGTA (≈1.7-fold), suggesting that Ca^{2+} inhibited Ca^{2+} channel activity in WT.[20] In contrast, the responses of *AC5*$^{-/-}$ myocytes to ISO were essentially the same in all three conditions (≈1.5-fold), suggesting that Ca^{2+}-mediated inhibition of Ca^{2+} channel activity was markedly diminished in *AC5*$^{-/-}$. These results suggest that intracellular Ca^{2+} can inhibit β-AR–mediated activation of Ca^{2+} channels, presumably through directly inhibiting cardiac AC activity,[20] and that type 5 AC is a major target of this inhibition (Figure 7B).

Discussion

We developed a mouse model with disruption of a major AC isoform (type 5) in the heart. It was predictable that increases in cardiac function in response to ISO would be diminished in *AC5*$^{-/-}$, as was demonstrated in this study. Indeed, the decrease in cardiac responsiveness to ISO in vivo paralleled the data in vitro on AC activity. Because overexpression of type 5 AC in the heart enhanced cardiac function,[11] we had expected that baseline cardiac function and HR would be reduced in *AC5*$^{-/-}$, which was not observed. Despite the decrease in AC activity, basal cardiac function and HR were not decreased in *AC5*$^{-/-}$. Actually, HR was significantly elevated in conscious *AC5*$^{-/-}$. Although we do not know all the mechanisms that contribute to the increase in cardiac rate, we propose at least three mechanisms that are impaired in *AC5*$^{-/-}$: muscarinic inhibition of AC activity, baroreflex restraint of HR, and Ca^{2+}-mediated inhibition of AC activity.

Because the elevated HR was not likely attributable to enhanced sympathetic tone, ie, sympathetic responses were attenuated in *AC5*$^{-/-}$ in both in vivo and in vitro experiments, we hypothesized that it was attributable, at least in part, to the loss of parasympathetic inhibition, because type 5 AC is a major Gi-inhibitable isoform in the adult heart.[17,18] To confirm this, we demonstrated that muscarinic stimulation, which inhibits cardiac function and HR, was attenuated in *AC5*$^{-/-}$ both in the presence and absence of enhanced β-AR stimulation with ISO. Conversely, atropine increased HR in WT but not in *AC5*$^{-/-}$, supporting the concept that the higher baseline HR was attributable to the loss of parasympathetic restraint. Furthermore, we demonstrated that arterial baroreflex slowing of HR, which occurs through parasympathetic nerves, was also blunted in *AC5*$^{-/-}$. Therefore, at any given arterial pressure, there is less baroreflex restraint, resulting in elevated HR. Taken together, these data provide convincing evidence in vivo that type 5 AC exerts a major role in parasympathetic regulation of cardiac function in addition to its key role in sympathetic regulation, which has been recognized for some time. Thus, AC-mediated parasympathetic modulation of ventricular function and atrial function, ie, HR, must be considered along with the more widely recognized mechanisms involving muscarinic modulation of K^+ channel activity[30,38] and muscarinic regulation at the level of membrane receptors, or Gi. To support this conclusion, the K^+ current in atrial myocytes and the expression of G proteins, β-ARK, muscarinic receptor type 2, and β- and α_1-AR were not altered in *AC5*$^{-/-}$. Finally, it is also conceivable that the impaired Ca^{2+} inhibition of AC also contributes to the increased HR at baseline.

To conclude that tachycardia in *AC5*$^{-/-}$ was attributable to the loss of parasympathetic restraint, it is important to rule out the possibility that some other compensatory pathway did not cause the tachycardia. This possibility is unlikely for several reasons. First, the increase in HR is not compensatory but is actually opposite the prediction that reduced contractility and HR would be expected from disruption of AC. Although unlikely, it is still possible that the resetting autonomic activity in the brain, or some mechanism at the level of Ca^{2+} channels, could be involved. Type 5 AC is also located in the striatum of the brain, and disrupting this isoform of AC does alter dopaminergic transmission in the brain.[39,40] However, it is more likely that parasympathetic stimulation leads to activation of muscarinic receptors and Gi to inhibit type 5 AC in the heart, which results in restraint on baseline HR. In the absence of type 5 AC, this restraint is lost and HR rises, as we observed in the *AC5*$^{-/-}$ mice in this investigation. It is important to note that the bradycardia resulting from pharmacologic muscarinic inhibition with ACh was attenuated in *AC5*$^{-/-}$, indicating that the mechanism is localized to the heart and does not reside in the CNS. In additional support of this conclusion are the complementary in vitro data from cardiac membranes. HR is thought to be regulated at the level of the muscarinic receptor, or Gi, or GIRK.[36] In the present investigation, coupling between muscarinic receptors and GIRK was not altered in *AC5*$^{-/-}$. In view of the major alteration in muscarinic control in *AC5*$^{-/-}$, we conclude that cardiac rate of contractility is also regulated at the level of AC. In support of this concept, a recent study suggested that muscarinic inhibition of β_1-AR stimulation may occur at the level of cAMP[41] and that β_1-AR and type 5 AC are located in the same subcellular fraction.[42]

In cardiac muscle, Ca^{2+} influx through the L-type Ca^{2+} channel is the primary pathway for initiation and maintenance and for the modulation of contractility by catecholamines. The increase in I_{Ca} by the β-adrenergic agonist ISO occurs via a cascade of events leading to protein kinase A–mediated phosphorylation of components associated with the Ca^{2+} channel. In turn, cardiac AC is regulated negatively by low concentrations of Ca^{2+}.[19,20] This mechanism was also impaired in *AC5*$^{-/-}$. The extent to which this mechanism is impaired in *AC5*$^{-/-}$ must be interpreted cautiously, because small changes in experimental conditions can influence the magnitude of the results. Our finding suggested that under physiological conditions, an increase in Ca^{2+} entry and inhibition of type 5 AC, leading to decreased phosphorylation and thus activity of the Ca^{2+} channel, can work synergistically to provide an intrinsic feedback mechanism for cellular Ca^{2+} homeostasis. Thus, because of the lack of Ca^{2+}-inhibitable type 5 AC in *AC5*$^{-/-}$, this negative feedback inhibition of the L-type Ca^{2+} channel may be lost. This loss may account for, at least in part, the maintained cardiac function in *AC5*$^{-/-}$. It is also important to consider the possibility that differences in SR loading and Ca^{2+} handling may have affected the response to ISO. However, in previous studies,[20,37] we found in mouse ventricular myocytes that AC activity and subsequent cAMP synthesis, which modulate Ca^{2+} channel activity, are regulated by the Ca^{2+} entering through the Ca^{2+} channel rather than by Ca^{2+} released from the SR stores.

Another consideration is potential changes in calmodulin levels, which could regulate Ca^{2+}-dependent Ca^{2+} channel inactivation.[43] However, *AC5*$^{-/-}$ mice did not exhibit changes in Ca^{2+} channel amplitude or inactivation time course. Furthermore, calmodulin content assessed by Western blotting did not change in the *AC5*$^{-/-}$ (data not shown).

In summary, because type 5 AC is the major AC isoform expressed in the adult mouse heart, it was surprising to find no effect on baseline cardiac function but rather an increase in HR despite reduced baseline AC activity. Both the increased basal HR and blunted baroreflex-mediated bradycardia may be related to a loss of parasympathetic restraint and reduced Ca^{2+} regulation of AC. Other mechanisms, not yet identified, may also play a role in mediating these results. Thus, type 5 AC regulates cardiac inotropy and chronotropy through the parasympathetic arm of the autonomic nervous system as well as through the sympathetic arm. Therefore, these new mechanisms for regulation of parasympathetic/sympathetic interactions and Ca^{2+}-mediated regulation conveyed by this specific AC isoform in the heart will likely have broad significance for the understanding of the pathophysiology and treatment of heart failure as well as in normal cardiac regulation.

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References

1. Murray KJ, Reeves ML, England PJ. Protein phosphorylation and compartments of cyclic AMP in the control of cardiac contraction. *Mol Cell Biochem*. 1989;89:175–179.
2. Federman AD, Conklin BR, Schrader KA, Reed RR, Bourne HR. Hormonal stimulation of adenylyl cyclase through Gi-protein $\beta\gamma$ subunits. *Nature*. 1992;356:159–161.
3. Ishikawa Y. Regulation of cAMP signaling by phosphorylation. *Adv Second Messengers Phosphoprotein Res*. 1998;32:99–120.
4. Hanoune J, Defer N. Regulation and role of adenylyl cyclase isoforms. *Annu Rev Pharmacol Toxicol*. 2001;41:145–174.
5. Caulfield MP. Muscarinic receptors: characterization, coupling and function. *Pharmacol Ther*. 1993;58:319–379.
6. Nagata K, Communal C, Lim CC, Jain M, Suter TM, Eberli FR, Satoh N, Coluci WS, Apstein CS, Liao R. Altered β-adrenergic signal transduction in nonfailing hypertrophied myocytes from Dahl salt-sensitive rats. *Am J Physiol Heart Circ Physiol*. 2000;279:H2502–H2508.
7. Kudej RK, Iwase M, Uechi M, Vatner DE, Oka N, Ishikawa Y, Shannon RP, Bishop SP, Vanter SF. Effects of chronic β-adrenergic receptor stimulation in mice. *J Mol Cell Cardiol*. 1997;29:2735–2746.
8. Vatner DE, Sato N, Galper JB, Vatner SF. Physiological and biochemical evidence for coordinate increases in muscarinic receptors and Gi during pacing-induced heart failure. *Circulation*. 1996;94:102–107.
9. Lipskaia L, Defer N, Esposito G, Hajar I, Garel MC, Rockman HA, Hanoune J. Enhanced cardiac function in transgenic mice expressing a Ca^{2+}-stimulated adenylyl cyclase. *Circ Res*. 2000;86:795–801.
10. Gao MH, Lai NC, Roth DM, Zhou J, Zhu J, Anzai T, Dalton N, Hammond HK. Adenylylcyclase increases responsiveness to catecholamine stimulation in transgenic mice. *Circulation*. 1999;99:1618–1622.
11. Tepe NM, Lorenz JN, Yatani A, Dash R, Kranias EG, Dorn GW, Liggett SB. 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. *Biochemistry*. 1999;38:16706–16713.

12. Ishikawa Y, Homcy CJ. The adenylyl cyclases as integrators of transmembrane signal transduction. *Circ Res*. 1997;80:297–304.
13. Tang WJ, Hurley JH. Catalytic mechanism and regulation of mammalian adenylyl cyclases. *Mol Pharmacol*. 1998;54:231–240.
14. Hurley JH. Structure, mechanism, and regulation of mammalian adenylyl cyclase. *J Biol Chem*. 1999;274:7599–7602.
15. Tobise K, Ishikawa Y, Holmer SR, Im MJ, Newell JB, Yoshi H, Fujita M, Susannie EE, Homcy CJ. Changes in type VI adenylyl cyclase isoform expression correlate with a decreased capacity for cAMP generation in the aging ventricle. *Circ Res*. 1994;74:596–603.
16. Espinasse I, Iourgenko V, Defer N, Samson F, Hanoune J, Mercadier JJ. Type V, but not type VI, adenylyl cyclase mRNA accumulates in the rat heart during ontogenic development: correlation with increased global adenylyl cyclase activity. *J Mol Cell Cardiol*. 1995;27:1789–1795.
17. Taussig R, Iniguez-Lluhi JA, Gilman AG. Inhibition of adenylyl cyclase by Giα. *Science*. 1993;261:218–221.
18. Dessauer CW, Tesmer JJ, Sprang SR, Gilman AG. Identification of a Giα binding site on type V adenylyl cyclase. *J Biol Chem*. 1998;273:25831–25839.
19. Cooper DMF, Mons N, Karpen JW. Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature*. 1995;374:421–424.
20. Sako H, Sperelakis N, Yatani A. Ca^{2+} entry through cardiac L-type Ca^{2+} channels modulates β-adrenergic stimulation in mouse ventricular myocytes. *Pflügers Arch*. 1998;435:749–752.
21. Ishikawa Y, Sorota S, Kiuchi K, Shannon RP, Komamura K, Katsushika S, Vatner DE, Vatner SF, Homcy CJ. Downregulation of adenylylcyclase types V and VI mRNA levels in pacing-induced heart failure in dogs. *J Clin Invest*. 1994;93:2224–2229.
22. Boyajian CL, Garritsen A, Cooper DMF. Bradykinin stimulates Ca^{2+} mobilization in NCB-20 cells leading to direct inhibition of adenylylcyclase: a novel mechanism for inhibition of cAMP production. *J Biol Chem*. 1991;266:4995–5003.
23. Cooper DMF. Regulation of Ca^{2+}-sensitive adenylyl cyclases by calcium ion in vitro and in vivo. *Methods Enzymol*. 1994;238:71–81.
24. Iwase M, Uechi M, Vatner DE, Asai K, Shannon RP, Kudej RK, Wagner TE, Wight DC, Patrick TA, Ishikawa Y, Homcy CJ, Vatner SF. Cardiomyopathy induced by cardiac Gsα overexpression. *Am J Physiol*. 1997;272:H585–H589.
25. Uechi M, Asai K, Osaka M, Smith A, Sato N, Wagner TE, Ishikawsa Y, Hayakawa H, Vatner DE, Shannon RP, Homcy CJ, Vatner SF. Depressed heart rate variability and arterial baroreflex in conscious transgenic mice with overexpression of cardiac Gsα. *Circ Res*. 1998;82:416–423.
26. Hart CY, Burnett JC, Redfield MR. Effects of avertin versus xylazine-ketamine anesthesia on cardiac function in normal mice. *Am J Physiol Heart Circ Physiol*. 2001;281:H1938–H1945.
27. Roth DM, Swaney JS, Dalton ND, Gilpin EA, Ross JJ. Impact of anesthesia on cardiac function during echocardiography in mice. *Am J Physiol Heart Circ Physiol*. 2002;282:H2134–H2140.
28. Gaudin C, Ishikawa Y, Wight DC, Mahdavi V, Nadal-Ginard B, Wanger TE, Vatner DE, Homcy CJ. Overexpression of Gsα protein in the hearts of transgenic mice. *J Clin Invest*. 1995;95:1676–1683.
29. Yatani A, Brown AM, Schwartz A. Bepridil block of cardiac calcium and sodium channels. *J Pharmacol Exp Ther*. 1986;237:9–17.
30. Yatani A, Codina J, Brown AM, Birnbaumer L. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein Gk. *Science*. 1987;235:207–211.
31. Masaki H, Sato Y, Luo W, Kranias EG, Yatani A. Phospholamban deficiency alters inactivation kinetics of L-type Ca^{2+} channels in mouse ventricular myocytes. *Am J Physiol*. 1997;272:H606–H612.
32. Mitarai S, Reed TD, Yatani A. Changes in ionic currents and β-adrenergic receptor signaling in hypertrophied myocytes overexpressing Gαq. *Am J Physiol Heart Circ Physiol*. 2000;279:H139–H148.
33. Breitwieser GE, Szabo G. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature*. 1985;317:538–540.
34. Pfaffinger PJ, Martin JM, Hunter DD, Nathanson NM, Hille B. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature*. 1985;317:536–538.
35. Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE. The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature*. 1987;325:321–326.
36. Wickman K, Nemec J, Gendler SJ, Clapham DE. Abnormal heart rate regulation in GIRK4 knockout mice. *Neuron*. 1998;20:103–114.
37. Sako H, Green SA, Kranias EG, Yatani A. Modulation of cardiac Ca^{2+} channels by isoproterenol studied in transgenic mice with altered SR Ca^{2+} content. *Am J Physiol*. 1997;273:C1666–C1672.
38. Wickman K, Clapham DE. Ion channel regulation by G proteins. *Physiol Rev*. 1995;75:865–885.
39. Lee KW, Hong JH, Choi IY, Che Y, Lee JK, Yang SD, Song CW, Kang HS, Lee JH, Noh JS, Shin HS, Han PL. Impaired D2 dopamine receptor function in mice lacking type 5 adenylyl cyclase. *J Neurosci*. 2002;22:7931–7940.
40. Iwamoto T, Okumura S, Iwatsubo K, Kawabe J, Ohtsu K, Sakai I, Hashimoto Y, Izumitani A, Sango K, Ajiki K, Toya Y, Umemura S, Goshima Y, Arai N, Vatner SF, Ishikawa Y. Motor dysfunction in type 5 adenylyl cyclase-null mice. *J Biol Chem*. 2003;278:16936-16940.
41. Aprigliano O, Rybin VO, Pak E, Robinson RB, Steinberg SF. β_1- and β_2-adrenergic receptors exhibit differing susceptibility to muscarinic accentuated antagonism. *Am J Physiol*. 1997;272:H2726–H2735.
42. Rybin VO, Xu X, Lisanti MP, Steinberg SF. Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. *J Biol Chem*. 2000;275:41447–41457.
43. Peterson BZ, DeMaria CD, Adelman JP, Yue DT. Calmodulin is the Ca^{2+} sensor for Ca^{2+}-dependent inactivation of L-type calcium channels. *Neuron*. 1999;22:549–558.

Type 5 Adenylyl Cyclase Disruption Alters Not Only Sympathetic But Also Parasympathetic and Calcium-Mediated Cardiac Regulation

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ONLINE DATA SUPPLEMENT

Expanded Materials and Methods

Generation of Knockout Mice

The targeting construct was prepared by ligating a 2.2-kb fragment from the 5' end of the type 5 AC gene, which contains the exon with the first translation initiation site (5'-arm); a fragment containing a neomycin resistance gene fragment driven by a phosphoglycerate kinase promoter; and a 7.0-kb fragment of the type 5 AC gene (3'-arm) into pBluscript II KS (Stratagene) [1]. Embryonic stem cells were transfected with 50 μg of linearized targeting vector by electroporation. Two clones were injected into C57BL/6 blastocysts, and chimeras were obtained. These chimeras successfully allowed germ-line transmission and were crossed with C57BL/6 females. F1-heterozygous offspring were then interbred to produce homozygous mutations. All mice were 129/SvJ-C57BL/6 mixed background littermates from F1 heterozygote crosses. All experiments were performed in 4-6 month old homozygous $AC5^{-/-}$ and wild type (WT) littermates. This study was approved by the Animal Care and Use Committee at New Jersey Medical School.

Radioligand Binding Assays and Western Blotting

Radioligand binding assays for β-AR were conducted using the above membrane preparations and ^{125}I-cyanopindolol as previously described [2]. Western blotting for type 5 AC, Gsα, Giα, Gqα, Gβγ, β_1-adrenergic receptor (β_1-AR), β-adrenergic receptor kinase (β-ARK), and muscarinic receptor type 2 were conducted using either the membrane preparation or whole tissue homogenates. Western blotting was conducted using commercially available antibodies, except for type 5 AC antiserum, which was

raised against the 27-mer amino acids (NH2-IGHNPPHWGAERPFYNHLGGNQVSKEC-COOH) that was obtained from the C1b domain of AC5 (amino acid residues 638-662). This sequence is conserved among different animal species but shows a very low sequence homology (14.8%) to the corresponding domain of the type 6 AC (kindly provided by Dr. James E Tomlinson, Millennium Pharmaceuticals Inc., Cambridge, MA). Because of its high specificity to type 5 AC, it does not cross react to type 6 AC. Accordingly, $AC5^{-/-}$ showed a null, not reduced, expression of type 5 AC.

Electrophysiological Studies

Whole-cell currents were recorded using patch-clamp techniques as previously described [3-6]. Cell capacitance was measured using voltage ramps of 0.8 V/s from a holding potential of -50 mV. All experiments were performed at room temperature. Ca^{2+} channel currents (I_{Ca}) were measured with an external solution (mmol/L): $CaCl_2$ or $BaCl_2$ 2; $MgCl_2$, 1; tetraethyl ammonium chloride, 135; 4-aminopyridine, 5; glucose, 10 and HEPES, 10 (pH 7.3). The pipette solution contained (mmol/L): Cs-aspartate, 100; CsCl, 20; $MgCl_2$, 1; MgATP, 2; GTP, 0.5; EGTA, 5 or 1,2-bis (2-aminophenoxy)ethane - *N, N, N', N'* - tetraacetic acid (BAPTA), 10 and HEPES, 5 (pH 7.3). For potassium (K^+) channel current recordings, the external solution was normal Tyrode's solution (mmol/L): NaCl, 135; $CaCl_2$, 1.8; $MgCl_2$, 1; KCl, 5.4; glucose, 10; HEPES, 10 (pH 7.3). Nifedipine (10 μmol/L) was added to block L-type Ca^{2+} channel currents. The patch pipette solution contained (mmol/L): potassium aspartate, 110; KCl, 20; $MgCl_2$, 2; ATP, 2; GTP, 0.5; EGTA, 5; HEPES, 5 (pH 7.3).

References

1. Iwamoto T, Okumura S, Iwatsubo K, Kawabe J, Ohtsu K, Sakai I, Hashimoto Y, Izumitani A, Sango K, Ajiki K, Toya Y, Umemura S, Goshima Y, Arai N, Vatner SF, Ishikawa Y. Motor Dysfunction in Type 5 Adenylyl Cyclase-Null Mice. *J Biol Chem.* 2003;278:16936-16940.
2. Gaudin C, Ishikawa Y, Wight DC, Mahdavi V, Nadal-Ginard B, Wagner TE, Vatner DE, Homcy CJ. Overexpression of Gsα protein in the hearts of transgenic mice. *J Clin Invest.* 1995;95:1676-1683.
3. Yatani A, Brown AM, Schwartz A. Bepridil block of cardiac calcium and sodium channels. *J Pharmacol Exp Ther.* 1986;237:9-17.
4. Yatani A, Codina J, Brown AM, Birnbaumer L. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein G_k. *Science.* 1987;235:207-211.
5. Masaki H, Sato Y, Luo W, Kranias EG, Yatani A. Phospholamban deficiency alters inactivation kinetics of L-type Ca^{2+} channels in mouse ventricular myocytes. *Am J Physiol.* 1997;272:H606-H612.
6. Mitarai S, Reed TD, Yatani A. Changes in ionic currents and β-adrenergic receptor signaling in hypertrophied myocytes overexpressing Gαq. *Am J Physiol Heart Circ Physiol.* 2000;279:H139-H148.

Supplemental Figure 1 Legend

Comparison of histology in WT and *AC5*$^{-/-}$ heart. Note the normal architecture of the *AC5*$^{-/-}$ myocardium.

Supplementary Figure 1