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 vitro6 or in vivo7,8 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 (Ca2+).19 Therefore, we also examined the regulation of AC activity by Ca2+.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

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 treatment were attenuated in AC5−/− 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, neurally 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 (Ca2+)-mediated inhibition of AC activity and Ca2+ channel function were diminished in AC5−/−. Thus, AC5−/− attenuates sympathetic responsiveness and also impairs parasympathetic and Ca2+-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
AC-specific antibody.

cardiac membranes. isolated myocytes, and assessing AC activity in vitro in New Jersey Medical School (see the online supplement, available at This study was approved by the Animal Care and Use Committee at 28S ribosomal RNA probe was used as an internal control. 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 Partial structure of the type 5 AC gene (WT), KpnI; E, EcoRI; X, XhoI; A, ApaI; P, PstI; BS, BssHII; H, HindIII; RV, EcoRV; N, NcoI; and B, BamHI. 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 KO mouse heart compared with WT using type 5 AC-specific antibody.

HR in conscious mice, measuring Ca2+ 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 littersmates from F1 heterozygote crosses. All experiments were performed in 4- to 6-month-old homozygous AC5 KO and wild-type (WT) littersmates. 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 Ca2+ inhibition, the membranes were treated first with EGTA to extract the endogenous Ca2+ before the assay. Free Ca2+ 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 125I-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 (See the online data supplement for additional detail.)

Statistical Analysis

All data are reported as mean±SEM. Comparisons between AC5 KO 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 KO (Figure 1C). Type 5 AC cardiac protein was undetectable in AC5 KO (Figure 1D). The growth, general appearance, and heart size of the AC5 KO were similar to WT (Table). Normal cardiac architecture was demonstrated on light microscopy (see the online data supplement).
AC Activity Was Decreased in the Heart of AC5<sup>−/−</sup> In Vitro

AC activity was decreased in AC5<sup>−/−</sup> 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<sup>−/−</sup>, indicating that the only response to ISO was attenuated in AC5<sup>−/−</sup>. 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<sup>−/−</sup> (Figure 3B), indicating that muscarinic (Gi-induced) inhibition of the AC activity is markedly attenuated in AC5<sup>−/−</sup>.

Regulation of AC Activity by Free Ca<sup>2+</sup>

To investigate the modulation of AC activity by free Ca<sup>2+</sup>, we examined cAMP production in membranes from the hearts of WT and AC5<sup>−/−</sup> at different Ca<sup>2+</sup> 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<sup>2+</sup> as expected in WT. The Ca<sup>2+</sup> inhibition of AC activity was impaired in AC5<sup>−/−</sup>. The reduction in magnitude of inhibition was most apparent in AC5<sup>−/−</sup>, ie, in the submicromolar range of Ca<sup>2+</sup> (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<sup>−/−</sup>. The cardiac responses to intravenous ISO on LVEF in AC5<sup>−/−</sup> were attenuated as expected (Figure 4). However, baseline cardiac function was not different between WT and AC5<sup>−/−</sup> (LVEF: WT versus AC5<sup>−/−</sup>, 70±1.2% versus 70±1.5%, n=10 to 11; fractional shortening: WT versus AC5<sup>−/−</sup>, 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...
in AC5\(^{-/-}\) (Figure 4), suggesting that muscarinic inhibition of \(\beta\)-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\(\pm\)11 versus 613\(\pm\)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\(\pm\)11 versus 36\(\pm\)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, ex parasympathetic bradycardia was impaired in AC5−/− compared with WT and AC5+/−. These data suggest that reflex parasympathetic bradycardia is 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, Gβγ, Gβ, Gγ, and β-adrenergic receptor kinase (β-ARK) as well as α-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.

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. 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 Ca2+ Channel Activity and Response to ISO
Peak inward ICa amplitude (with 5 mmol/L EGTA in the pipette solution), normalized to cell capacitance (ICa 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 ICa at +10 mV was 21.9±1.4 and 21.0±1.4 ms for AC5−/− and WT, respectively. These data suggest that changes in AC activity did not directly influence Ca2+ channel density or inactivation kinetics. In previous studies, we have proposed that AC activity and subsequent cAMP synthesis, which modulate Ca2+ channel activity, are regulated by Ca2+ influx through the channel. We thus compared the effects of ISO on ICa using procedures designed to modulate the cytoplasmic Ca2+ concentration with two different Ca2+ chelators, EGTA and BAPTA, the latter of which have faster Ca2+ binding kinetics, and with the use of extracellular barium (Ba2+), which permeates the Ca2+ channel but does not trigger Ca2+ of the sarcoplasmic reticulum (SR). Figure 7A shows a typical example of the effect of ISO (1 μmol/L) on ICa 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 ICa 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 Ba2+ was used, the maximum response of the Ca2+ channel to ISO was significantly augmented (≈2.4-fold) compared with cells.
enhanced sympathetic tone, ie, sympathetic responses were not decreased in AC5−/− mice, as was demonstrated in this study. Indeed, the decrease in cardiac responsiveness to ISO would be diminished in AC5−/− mice. In the presence of ISO, peak Ica 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 Ica measured in myocytes dialyzed with EGTA or BAPTA and on Ba2+ 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.

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−/− mice, as was demonstrated in this study. Indeed, the decrease in cardiac responsiveness to ISO would be diminished in AC5−/− mice. Baseline 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−/− mice: muscarinic inhibition of cardiac AC activity, baroreflex restraint of HR, and Ca2+−mediated inhibition of cardiac 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.11,12 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 activity30,31 and muscarinic regulation at the level of membrane receptors, or G proteins. β-ARK, muscarinic receptor type 2, and β- and α1-AR were not altered in AC5−/−. Finally, it is also conceivable that the impaired Ca2+ inhibition of AC also contributes to the increased HR at baseline.

Figure 7. A, Effects of ISO on Ica 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 Ica 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 Ica density between WT and AC5−/− myocytes. There was no difference in control Ica density between WT and AC5−/− myocytes. In the presence of ISO, peak Ica 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 Ica measured in myocytes dialyzed with EGTA or BAPTA and on Ba2+ 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.
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 Ca2+ 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.99,100 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 receptors 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 β1-AR stimulation may occur at the level of cAMP41 and that β1-AR and type 5 AC are located in the same subcellular fraction.42

In cardiac muscle, Ca2+ influx through the L-type Ca2+ channel is the primary pathway for initiation and maintenance and for the modulation of contractility by catecholamines. The increase in ICa by the β-adrenergic agonist ISO occurs via a cascade of events leading to protein kinase A–mediated phosphorylation of components associated with the Ca2+ channel. In turn, cardiac AC is regulated negatively by low concentrations of Ca2+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 Ca2+ entry and inhibition of type 5 AC, leading to decreased phosphorylation and thus activity of the Ca2+ channel, can work synergistically to provide an intrinsic feedback mechanism for cellular Ca2+ homeostasis. Thus, because of the lack of Ca2+-inhibitable type 5 AC in AC5−/−, this negative feedback inhibition of the L-type Ca2+ 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 Ca2+ 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 Ca2+ channel activity, are regulated by the Ca2+ entering through the Ca2+ channel rather than by Ca2+ released from the SR stores.

Another consideration is potential changes in calmodulin levels, which could regulate Ca2+-dependent Ca2+ channel inactivation.43 However, AC5−/− mice did not exhibit changes in Ca2+ 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 Ca2+ 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 Ca2+-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.

Acknowledgments

This work was supported in part by NIH grants HL59729, HL61476, HL67724, HL65183, HL65182, HL69020, HL59139, AG14121, and HL33107 and by American Heart Association grants 9940187N, 9950673N, and 0020323U. Y. I. is a recipient of the Established Investigator Award of the American Heart Association.

References

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

Satoshi Okumura, Jun-ichi Kawabe, Atsuko Yatani, Gen Takagi, Ming-Chih Lee, Chull Hong, Jing Liu, Ikuyo Takagi, Junichi Sadoshima, Dorothy E. Vatner, Stephen F. Vatner and Yoshihiro Ishikawa

Circ Res. 2003;93:364-371; originally published online July 17, 2003;
doi: 10.1161/01.RES.0000086986.35568.63

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/93/4/364

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2003/08/22/93.4.364.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
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 ¹²⁵I-cyanopindolol as previously described ². Western blotting for type 5 AC, Gsα, Gtα, Gqα, Gβγ, β1-adrenergic receptor (β1-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


Supplemental Figure 1 Legend

Comparison of histology in WT and AC5⁻/⁻ heart. Note the normal architecture of the AC5⁻/⁻ myocardium.
Supplementary Figure 1

WT       AC5

A       B

100µm