Carvedilol reverses cardiac insufficiency in AKAP5 knockout mice by normalizing the activities of calcineurin and CaMKII

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Aims	Cardiac β-adrenergic receptors (β-AR) are key regulators of cardiac haemodynamics and size. The scaffolding protein A-kinase anchoring protein 79/150 (AKAP5) is a key regulator of myocardial signalling by β-ARs. We examined the function of AKAP5 in regulating cardiac haemodynamics and size, and the role of β-ARs and Ca^{2+}-regulated intracellular signalling pathways in this phenomenon.
Methods and results	We used echocardiographic, histological, genetic, and biochemical methods to examine the effect of ablation of AKAP5 on cardiac haemodynamics, size, and signalling in mice. $AKAP5^{-/-}$ mice exhibited enhanced signs of cardiac dilatation and dysfunction that progressed with age. Infusions of isoprenaline worsened cardiac haemodynamics in wild-type (WT) mice only, but increased the ratio of heart-to-body weight equally in WT and in $AKAP5^{-/-}$ mice. Mechanistically, loss of AKAP5 was associated with enhanced activity of cardiac calmodulin kinase II (CaMKII) and calcineurin (CaN) as indexed by nuclear factor of activated T-cell-luciferase activity. Loss of AKAP5 interfered with the recycling of cardiac β$_1$-ARs, which was mediated in part by CaN binding to AKAP5. Carvedilol reversed cardiac hypertrophy and haemodynamic deficiencies in $AKAP5^{-/-}$ mice by normalizing the activities of cardiac CaN and CaMKII.
Conclusions	These findings identify a novel cardioprotective role for AKAP5 that is mediated by regulating the activities of cardiac CaN and CaMKII and highlight a significant role for cardiac β-ARs in this phenomenon.
Keywords	AKAP • Cardiac hypertrophy • β-Adrenergic receptors • Calcineurin • Calmodulin kinase II

1. Introduction

Activation of cardiac β-adrenergic receptors (β-ARs) by catecholamines promotes acute elevation of cyclic AMP, which results in chronotropic and inotropic effects on the heart muscle. A-kinase anchoring proteins (AKAPs) bind protein kinase A (PKA) and other proteins and compartmentalize them into signalling complexes.[1] Among the more than 50 AKAPs that have been identified, AKAP5 has attracted a significant attention. AKAP5 is widely expressed in the periphery and plays a major role in forming discrete signalling networks. AKAP5 binds to PKA, protein kinase C and calcineurin (CaN), and other signalling molecules, such as adenylyl cyclase (AC) 5/6, voltage-gated L-type calcium channels (LTCCs), and post-synaptic density forming proteins such as

SAP97.[2,3] A relationship between cardiac β$_1$-ARs and AKAP5 was determined by immunohistochemical methods that revealed close association between post-synaptic β$_1$-AR and AKAP5.[4,5]

Studies in cardiac myocytes derived from $AKAP5^{-/-}$ mice indicated that AKAP5 exerted effects on the regulation of Ca^{2+} signalling and trafficking of the β$_1$-AR.[6,7] Nichols et al.[7] determined that β-AR-dependent increase in $[Ca^{2+}]_i$ and β-AR-mediated regulation of excitation–contraction coupling were eliminated in $AKAP5^{-/-}$ myocytes. The defect in β-AR-mediated activation of $[Ca^{2+}]_i$ was eventually traced to a subpopulation of LTCCs that were associated simultaneously with AKAP5 and cardiac caveolin 3.[7] Li et al.[8] determined that signalling by β$_1$-AR to cyclic AMP was more pronounced in neonatal $AKAP5^{-/-}$ neonatal mouse ventricular myocytes (NMVM). Sensitization of β$_1$-AR

signalling in AKAP5$^{-/-}$ cardiac myocytes resulted in increased cardiomyocyte contractility and size in response to β_1-agonists. In addition, intracellular trafficking of the β_1-AR was impaired in AKAP5$^{-/-}$ cardiac myocytes and restored by the expression of AKAP5. These findings indicate that AKAP5 was a major AKAP involved in regulating the physiology and trafficking of cardiac β_1-AR.[6–8]

In this study, we determined cardiac parameters in AKAP5$^{-/-}$ vs. wild-type (WT) AKAP5$^{+/+}$ mice to determine the effect of global ablation of AKAP5 on cardiac function. Our results indicate that AKAP5 was actually cardioprotective, because its deletion was associated with significant cardiac hypertrophy and insufficiency that were reversed by the mixed α- and β-blocker carvedilol. These results are the first to indicate a clear role for an AKAP in cardiac remodelling and in regulating cardiac haemodynamics.

2. Methods

2.1 Mice and rats

AKAP5$^{+/+}$ and AKAP5$^{-/-}$ mice have previously been described.[7,8] Animal experiments were performed according to protocols that were approved by the University of Tennessee-HSC Institutional Animal Care and Use Committee. Studies conformed to the 'Guide for Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). All mice were in the C57Bl6/J background and backcrossed >10 generations. Sprague−Dawley rats were used as the source of neonatal cardiac myocytes. Mice or rats were sacrificed by barbiturate overdose (pentobarbital, 150 mg/kg, i.p.).

2.2 Drug administration

One hour prior to placement of the Alzet mini-pump, each mouse received 0.125 mg/g of carprofen analgesic, i.p. Four-month-old mice were anaesthetized by inhalation of 2% isofluorane and the back area slightly posterior to the scapulae was shaved and wiped in betadine. A 0.5 cm skin incision was made and an Alzet model 2002 mini-pump that was filled with buffer (1% ascorbic acid in PBS) or isoprenaline (40 mg/kg/d) was inserted subcutaneously. The wound was closed with a 7 mm clip, followed by the application of triple antibiotic ointment. Pumps were removed 24 h before echocardiography following the same procedure used to implant them. Mouse chow containing placebo or carvedilol 1500 mg/kg was provided *ad libitum* to 4-month-old AKAP$^{+/+}$ or AKAP5$^{-/-}$ mice for 10 days.[9,10] Cyclosporine A (CsA, 10 mg/kg) was injected i.p. daily for 2 weeks to matched littermates of 4-month-old AKAP5$^{+/+}$ or AKAP5$^{-/-}$ mice of either sex.

2.3 Echocardiography and histology

Mice were anaesthetized with isofluorane (2%) and placed on the recording stage of a Vevo 2100 ultrasound and imaged using an 18−32 MHz MS400 transducer. The anterior chest was shaved and ultrasonic gel applied. Two-dimensional images were recorded in parasternal long- and short-axis projections with guided M-mode recordings at the midventricular level in both views. M-mode echocardiographic measurements were obtained before and after each treatment.

2.4 Immunohistochemistry

After echocardiography, mice or rats were sacrificed by barbiturate overdose. Hearts were excised, cleaned, and embedded in tissue freezing medium at -40°C and sectioned. Eight-micron slices were adhered to silanated glass slides and stained with haematoxylin−eosin or Masson trichrome. Other tissue sections were permeabilized in 0.2% Triton X-100 in PBS for 10 min at 4°C, incubated with anti-sarcomeric α-actinin and then incubated with a 1 : 100 dilution of rhodamine-conjugated goat anti-mouse (EMD Biosciences, San Diego, CA, USA), and analysed by immunocytochemistry.

2.5 Confocal recycling microscopy protocol

Cardiac myocytes were prepared from 1-day-old Sprague−Dawley rats and cultured on collagen-coated cover slips as described.[5,6,8] Myocytes were infected for 6 h with adenovirus expressing WT FLAG-tagged β_1-AR, WT FLAG-tagged β_2-AR, or activated CaN (JMAd-03, Seven Hills Bioreagents, Cincinnati, OH, USA) at multiplicity of infection (m.o.i.) of 100. Myocytes were washed and cultured for an additional 24−36 h before use. Cardiomyocytes were incubated with Cy3-labelled anti-FLAG IgG (5−10 μg/mL) and with 1 μM of FK-506 or buffer (1% DMSO) for 1 h at 37°C. HEK-293 cells stably expressing 1.2 pmol/mg protein of FLAG-tagged β_1-AR were transiently transfected with pEGFP-N1, AKAP79(Δ321−360) in pEGFP-N1, or PVIVIT in pEGFP-N1 for 2 days and then used.[8,11,12] Internalization and recycling were conducted as described,[5,6,8] followed by confocal microscopy using Cy3 (pseudo-red, for β-AR), DAPI (pseudo-violet, for nuclei), and GFP (pseudo-green) laser settings of the Olympus FluoView™ FV-1000 confocal microscope.

2.6 Measurement of the activity of luciferase-linked to nuclear factor of activated T-cells

AKAP5$^{-/-}$ mice were bred with mice that express luciferase upon the activation of nuclear NFAT (nuclear factor of activated T-cells; Jackson Laboratories, stock 013781). Hearts from AKAP5$^{+/+}$/NFAT-luc or AKAP5$^{-/-}$/NFAT-luc were excised from age-matched mice and fresh frozen with liquid N_2. Hearts were powdered and then extracted in $1\times$ luciferase lysis buffer (Promega) with protease inhibitors by three freeze/thaw cycles. Clarified cardiac lysates were used to measure the enzymatic activity of luciferase by a commercial kit (Luciferase Assay System, Promega Corp.). Light intensity was measured with a Turner-20 luminometer over 10 s and expressed as relative light units (RLUs) over 10 s/mg protein.

2.7 CaN activity assay

Phosphatase activity was measured using the CaN cellular activity kit (Enzo Life sciences), according to manufacturer's instructions. Cardiac extracts from either three AKAP5$^{+/+}$ or three AKAP5$^{-/-}$ mice were prepared, and CaN activity was measured as the dephosphorylation rate of a synthetic phosphopeptide substrate (RII peptide). The amount of PO_4 released was determined colorimetrically with the BIOMOL GREEN™ reagent.

2.8 Measurement of the activity of cardiac calmodulin kinase II

Calmodulin kinase II (CaMKII) activity in cardiac homogenates was assayed in a total volume of 25 μL with the use of a biotinylated CaMKII peptide (SignaTect™ CaMKII kit, Promega). Hearts were powdered and extracted by three freeze/thaw cycles in extraction buffer [20 mM Tris−HCL, (pH 8.0), 2 mM EDTA and 2 mM EGTA, and 2 mM dithiothreitol] with protease inhibitors. The assay contained 50 mM Tris (pH 7.4), 20 μM biotinylated CaMKII substrate, 0.1 mM ATP ([γ-^{32}P]ATP = 1 μCi per assay), 10 mM magnesium acetate, and 0.5 mM dithiothreitol. CaMKII activity was measured in the presence of $\pm$ 1 mM $CaCl_2$ and $\pm$ 1 μM calmodulin. The reaction was initiated by adding 10 μg of the homogenate, followed by incubation for 3 min at 30°C. Incorporation of ^{32}P in the peptide substrate was determined following the manufacturer's instructions. Cardiac homogenates prepared from age-matched AKAP5$^{+/+}$ or AKAP5$^{-/-}$ mice were subjected to immunoblotting with anti-δCaMKII (Santa Cruz) or anti-phospho (Thr^{286}) CaMKII (Cell Signaling Technology), followed by densitometric scanning of each band to determine optical density ratios.

2.9 Immunoprecipitation

CaNα in pcDNA3.0 myc/His was ectopically expressed in HEK-293 cells with either empty p-EGFP-N1 or with AKAP5(321−360)-GFP.[11] Cells were lysed and equal amounts of protein were subjected to affinity-purification on anti-AKAP5-IgG or pre-immune IgG beads. Then, equal volumes of eluate were probed for co-immunoprecipitation (IP) of CaNα by western blotting using anti-Myc (9E10) antibody (Santa Cruz Biotechnology) as described.

2.10 Statistical analysis

All data are expressed as mean $\pm$ SE. The significance of differences between baseline and drug treatment within same genetic species and between pre-carvedilol and post-carvedilol echocardiographic values within the group was evaluated by Student's *t*-test. To evaluate differences in haemodynamic parameters among groups of transgenic mice, multigroup comparisons were made by two-way ANOVA followed by the Bonferroni *post hoc* test, using the Graph Pad Prism™ software. In addition, two-way ANOVA with Bonferroni correction for multiple comparisons was used to evaluate the morphometric measurements and enzymatic activities. Statistical significance was defined as $P < 0.05$.

3. Results

3.1 Ablation of AKAP5 leads to ventricular hypertrophy

$AKAP5^{-/-}$ mice did not display obvious anatomical, growth, or productive changes.[7] However, deletion of AKAP5 resulted in progressive ventricular hypertrophy (*Figure 1A* and *B*). Histological sections confirmed enlarged left ventricular (LV) chambers in 3-month-old $AKAP5^{-/-}$ compared with $AKAP5^{+/+}$ mice and the enlargement progressed with age (*Figure 1A*). Body weights and left tibial lengths were not different, but there was a ~16% increase ($P < 0.05$) in the ratio of heart-to-body weight between $AKAP5^{-/-}$ and $AKAP5^{+/+}$ mice (*Figure 1B*). These differences were apparent when heart weights were normalized to body weight showing a 16% increase in the ratios at 4 months ($P < 0.05$). Cross-sectional area of cardiac cells in $AKAP5^{-/-}$ mice at 4 months ($679 \pm 100\ \mu m^2$) was significantly larger than the corresponding parameter ($439 \pm 103\ \mu m^2$) in $AKAP5^{+/+}$ littermates (*Figure 1C* and *D*). In addition, hearts from $AKAP5^{-/-}$ mice manifested ventricular myocyte interstitial fibrosis and increased collagen deposition (*Figure 1E* and *F*). Deletion of AKAP5 induced marked up-regulation in the expression of molecular markers of hypertrophy,[13] such as atrial and brain natriuretic peptides, and the ratio of β- to α-MHC (*Figure 1G*).

3.2 Loss of AKAP5 leads to compromised cardiac performance

Cardiac function was monitored by echocardiography in anaesthetized mice (*Figure 2A*). Loss of AKAP5 led to compromised cardiac function (*Figure 2B−F*). There was an age-dependent proportional decrease in systolic performance assessed as per cent ejection fraction from 65% in 4-month-old $AKAP5^{+/+}$ animals to 41% in matched $AKAP5^{-/-}$ littermates ($P < 0.01$). In addition, stroke volumes were significantly reduced ($P < 0.05$) in $AKAP5^{-/-}$ mice when compared with age-matched $AKAP5^{+/+}$ littermates (*Figure 2E*). Declines in cardiac function were associated with ventricular dilatation and a progressive increase in LV end-systolic and -diastolic dimensions (*Figure 2C* and *D*). An echocardiographic estimate of LV volume and necroscopic evaluation of heart-to-body weight indicated that loss of AKAP5 was associated with eccentric hypertrophy and dilatation.[14]

3.3 AKAP5 mice are resistant to stress-induced cardiac hypertrophy elicited by isoprenaline

Infusions of isoprenaline produce significant haemodynamic insufficiency, cardiac hypertrophy, and remodelling that is associated with significant apoptosis.[15,16] To determine the role of AKAP5 in β-AR-induced cardiomyopathy, isoprenaline was infused for 10 days into $AKAP5^{-/-}$ and $AKAP5^{+/+}$ mice littermates (*Figure 3A*). In WT $AKAP5^{+/+}$ mice, isoprenaline led to $-20 \pm 8\%$ reduction in the ejection fraction ($P < 0.05$) and significantly increased systolic LV volume by $24 \pm 6\%$ without affecting heart rate (*Figure 3A*).[17] In contrast, isoprenaline-mediated changes in cardiac function in $AKAP5^{-/-}$ mice were not statistically significant (*Figure 3A*). However, isoprenaline increased in the ratio of heart-to-body weight in $AKAP5^{+/+}$ or in $AKAP5^{-/-}$ mice by 1.5 ± 0.18-fold ($P < 0.05$ compared with no drug treatment; *Figure 3B*). Isoprenaline also increased the expression of hypertrophic markers, such as β-MHC, collagen-1a, and collagen-3a, by more than five-fold in $AKAP5^{+/+}$ and $AKAP5^{-/-}$ mice (*Figure 3C*).

3.4 Role of Ca^{2+}/CaM downstream signalling to CaN and CaMKII in haemodynamic dysfunction of $AKAP5^{-/-}$ mice

Nichols *et al.*[7] reported that β-AR-mediated activation of $[Ca^{2+}]_i$ via LTCC was impaired in $AKAP5^{-/-}$ cardiac myocytes. Aberrant handling of Ca^{2+} in cardiomyocytes affects the activity of the Ca^{2+}-binding protein CaM, which regulates the activities of Ca^{2+}/CaM-activated protein phosphatase 2B or CaN and CaMKII.[18,19] Hyperactive CaN or CaMKII is associated with cardiac hypertrophy.[18−20] To determine the effect of gene deletion of AKAP5 on the activity of cardiac CaN, $AKAP5^{-/-}$ mice were crossed with mice that expressed luciferase under the control of the NFAT nuclear transcription factor.[21] This approach is used to determine the activity of CaN *in vivo* because CaN dephosphorylates and activates cytosolic NFAT.[22] The activity of luciferase in cardiac extract from 4-month-old $AKAP5^{+/+}$-NFAT-Luc$^{Tg/?}$ mice was 620 ± 80 RLU/min/mg (*Figure 4A*). In age- and sex-matched AKAP5 knockout mice ($AKAP5^{-/-}$-NFAT-Luc$^{Tg/?}$ mice), the activity of CaN as indexed by luciferase increased by an additional three-fold ($P < 0.001$). Similarly, total CaN phosphatase activity (okadaic acid-resistant and EGTA-sensitive) in $AKAP5^{-/-}$ cardiac extracts was significantly higher ($P < 0.05$) than in $AKAP5^{+/+}$ extracts (0.40 ± 0.08 vs. 0.27 ± 0.06 pmol PO_4 released/μg protein, respectively; *Figure 4B*).

Extracts from hearts of age- and sex-matched 4-month-old $AKAP5^{+/+}$ and $AKAP5^{-/-}$ mice were compared for activity of CaMKII in the absence or presence of CaM and Ca^{2+} (*Figure 4C*). Ca^{2+} and CaM increased the enzymatic activity of CaMKII by 3 ± 0.5-fold in cardiac extracts from $AKAP5^{+/+}$ mice and by 6 ± 1.5-fold in cardiac extracts from $AKAP5^{-/-}$ mice ($P < 0.001$). Activity of CaMKII,[21] as indexed by the ratio of anti-phospho-CaMKII (Thr^{286}) over δCaMKII, was elevated in cardiac homogenates prepared from ventricles of $AKAP5^{-/-}$ mice vs. control mice (*Figure 4D*). Therefore, activities of CaN and CaMKII were both increased in hearts of $AKAP5^{-/-}$ mice.

3.5 Role of CaN and the CaN-binding site in AKAP5 on trafficking of β-AR in rodent cardiac myocytes

$β_1$-AR and $β_2$-AR are internalized in response to isoprenaline and recycle back into the cell membrane of cardiac myocytes or other
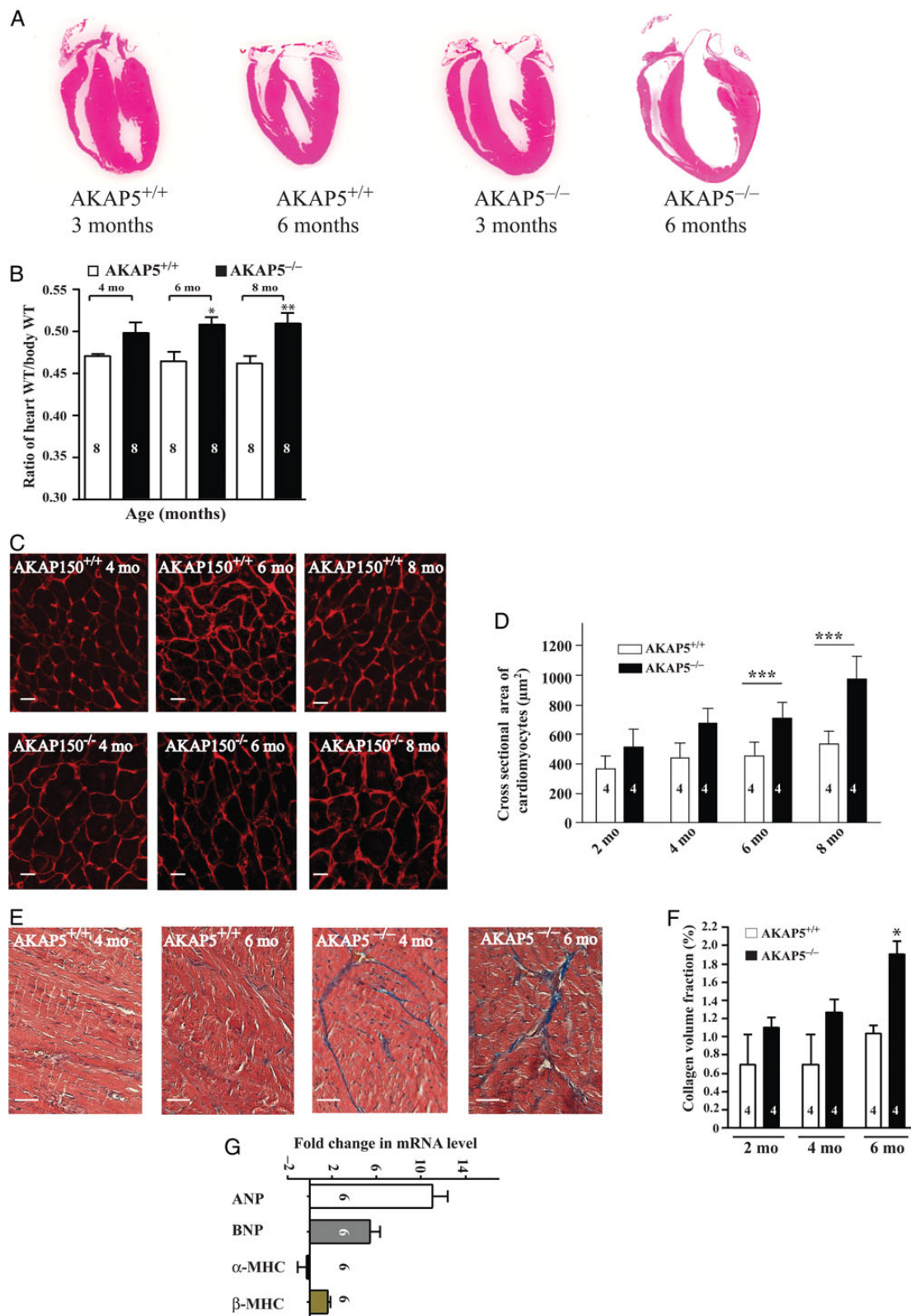
Figure 1 AKAP5$^{-/-}$ mice display a hypertrophic phenotype. (*A*) Histological sections of WT or AKAP5$^{-/-}$ hearts from 3- or 6-month-old mice. (*B*) Means ± SE of heart-to-body weight ratios between WT and AKAP5$^{-/-}$ mice at 4, 6, and 8 months of age. (*C* and *D*) Cell membrane staining (wheat germ agglutinin) and quantification of cross-sectional areas of heart cells from 4-, 6-, or 8-month-old WT and AKAP5$^{-/-}$ mice. Scale bars represent 25 μm. (*E* and *F*) Trichrome staining (fibrosis in blue) and quantification of fibrosis between 4- and 6-month-old WT and AKAP5$^{-/-}$ mice. Scale bars represent 20 μm. (*G*) RT-PCR analysis of hypertrophic marker gene expression in WT vs. AKAP5$^{-/-}$ mice at 4 months of age. Data were plotted as means ± SE. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ for either base in WT vs. base in knockout (KO) mice.
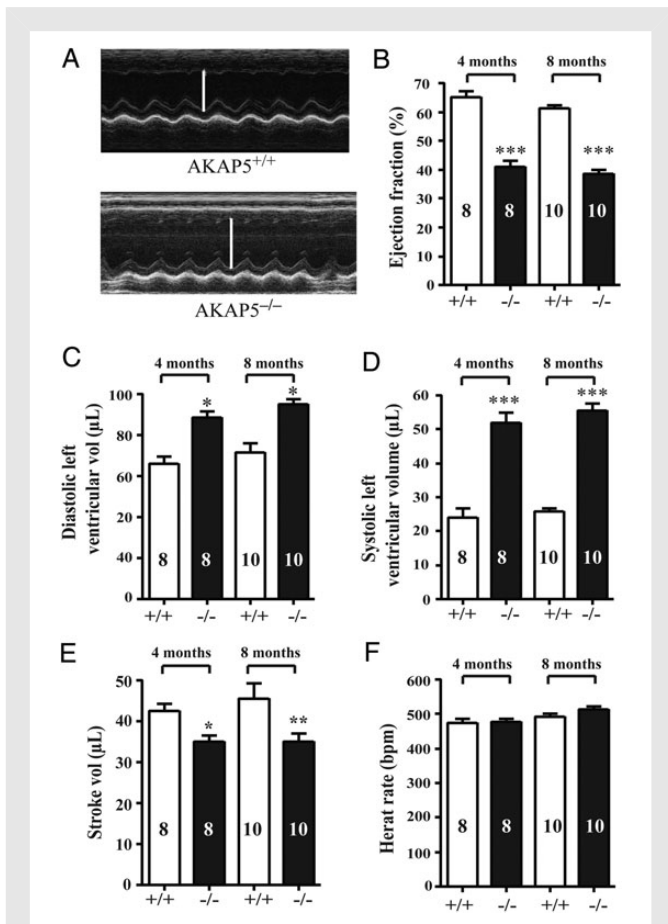
Figure 2 Cardiac echocardiography between WT and AKAP5$^{-/-}$ mice. (A) M-mode echocardiographic analysis of hearts from WT and AKAP5$^{-/-}$ mice. (B–F) Parameters of cardiac haemodynamic function in WT and AKAP5$^{-/-}$ mice. (B) Ejection fraction in %; (C) diastolic LV volume in μL; (D) systolic LV volume in μL; (E) stroke volume in μL and heart rate in bpm. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ represent the P-value for either base in WT vs. base in KO mice.

mammalian cells.[5,8] These results were replicated in rat cardiac myocytes that expressed either the β$_1$-AR or the β$_2$-AR (*Figure 5A* and *B*). WT β$_1$-AR or WT β$_2$-AR (pseudo-red colour) was expressed on the surface of the cardiomyocyte and then internalized in response to isoprenaline (*Figure 5A*, a and b, and *B*, p–q). Removal of isoprenaline promoted the recycling of internal β$_1$-AR or β$_2$-AR back into the myocardial cell membrane (*Figure 5A*, c–e and *B*, r–t). Since the activity of CaN was increased in hearts of AKAP5$^{-/-}$ mice, we wondered whether altered CaN activity somehow affected the trafficking of these β-AR. Therefore, the effect of enhancing or suppressing the activity of CaN on trafficking of the β$_1$-AR vs. the β$_2$-AR was determined (*Figure 5A* and *B*). In cardiac myocytes that expressed constitutively active CaN, WT β$_1$-AR or β$_2$-AR was internalized and recycled normally (*Figure 5A*, f–j, and *B*, u–y). Inhibition of CaN with FK-506 did not affect the internalization of WT β$_1$-AR in response to isoprenaline, but inhibited its recycling (*Figure 5A*, m–o). In contrast, FK-506 had no effect on the internalization and recycling of WT β$_2$-AR in rat cardiomyocytes (*Figure 5B*, a′–e′).

These findings are the first to show that inhibition of CaN differentially interfered with the trafficking of the β$_1$-AR, but how this might occur is unknown. As outlined earlier, deletion of AKAP5 also interfered with
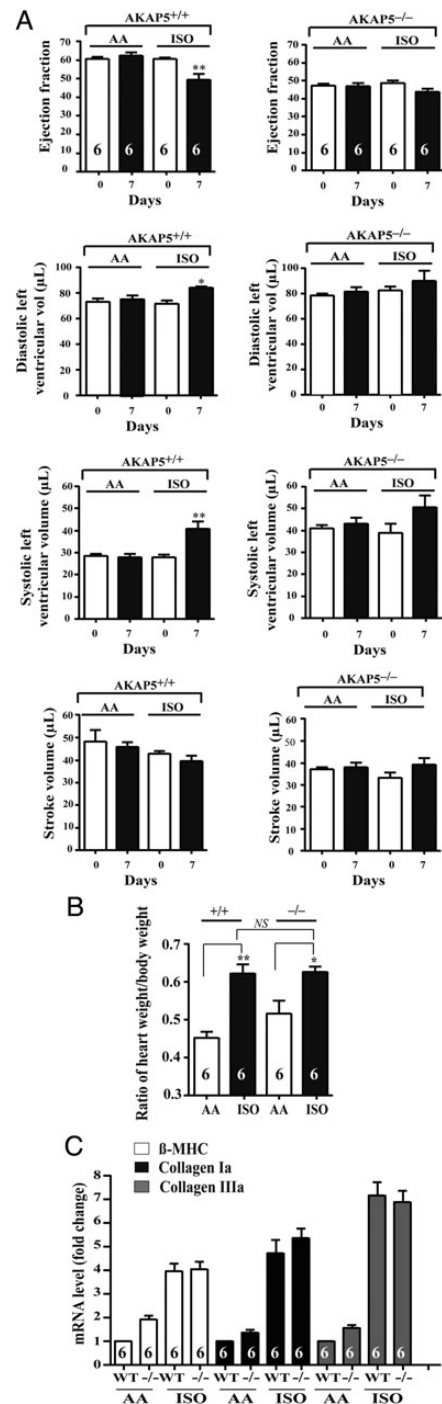

Figure 3 Effect of isoprenaline on cardiac haemodynamics and size in WT vs. AKAP5$^{-/-}$ mice. (A) Two-month-old WT and AKAP5$^{-/-}$ were echoed before the placement of the Alzet mini-osmotic pump. Buffer (1% ascorbic acid in PBS) or isoprenaline (100 mg/mL of buffer) was infused by a model 2002 mini-osmotic pump at a rate of 0.5 μL/h for 10 days. Then, the pumps were removed and the mice were echoed the next day. The data were plotted as means ± SE and then compared among different groups. (B) After the second echocardiography, mice were weighed and the heart was excised, weighed, and followed by extraction of RNA. (C) mRNA expression levels of hypertrophy markers were determined by quantitative RT-PCR. *$P < 0.05$ and **$P < 0.01$ represent the P-value of either base in WT vs. base in KO mice or isoprenaline in WT vs. isoprenaline in KO.
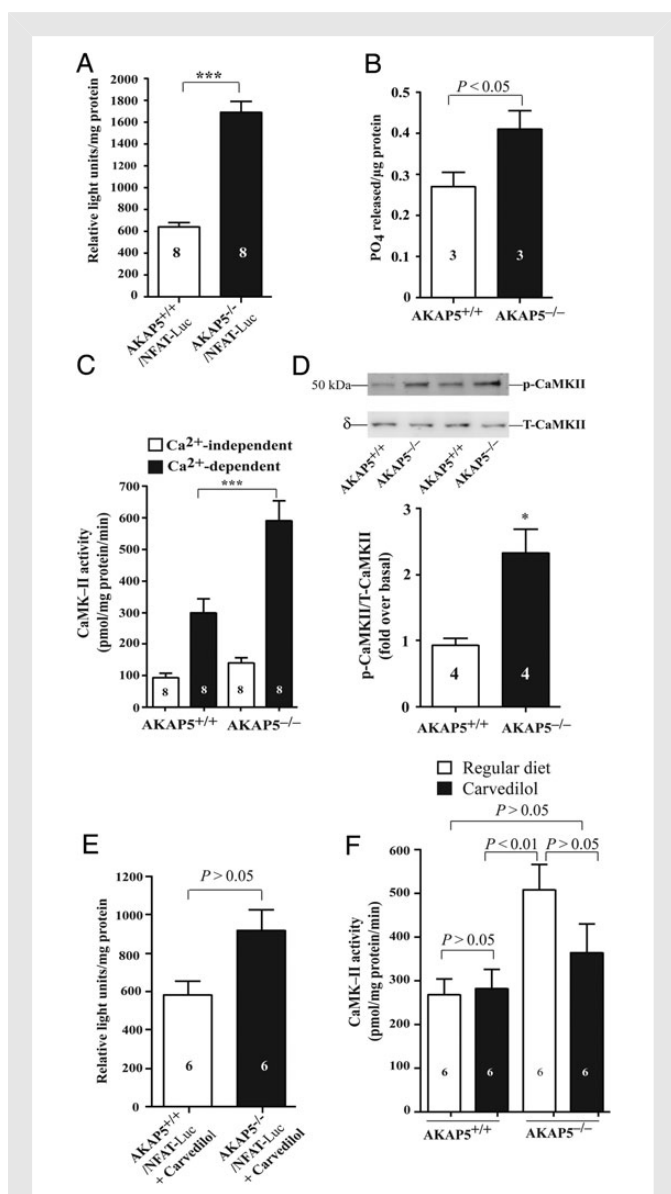
Figure 4 Comparison of the activities of CaN and CaMKII in hearts of transgenic mice that were fed regular chow and chow containing carvedilol. (*A*) Relative luc levels from cardiac protein extracts from 4-month-old mice containing NFAT-luc, in a WT AKAP5 (AKAP5$^{+/+}$) background or in a homozygous AKAP5 (AKAP5$^{-/-}$) background. All data were shown relative to background (no luc transgene), which were set a value of 1. (*B*) Comparison of the activity of CaN in cardiac extracts from control or AKAP5$^{-/-}$ mice. (*C*) CaMKII activity assayed in heart homogenate of 4-month-old (AKAP5$^{+/+}$) or AKAP5$^{-/-}$ mice in the absence of CaM/Ca^{2+} (open rectangle) or the presence of CaM/Ca^{2+} (closed rectangle). (*D*) Ratios of phospho-CaMKII (Thr^{286})/total δ-CaMKII in cardiac extracts prepared from WT or KO mice. (*E*) Four-month-old mice containing NFAT-luc, in a WT AKAP5 (AKAP5$^{+/+}$) background or in a homozygous AKAP5 (AKAP5$^{-/-}$) background, were fed regular diet or diet containing carvedilol for 10 days. Cardiac extracts were prepared and assayed for the activity of luciferase. (*F*) CaMKII activity was assayed in the presence of CaM/Ca^{2+} in heart homogenates prepared from 4-month-old WT or KO mice that were fed regular chow (open square) or chow containing carvedilol (closed square). In (*A*–*E*), the *P*-value represents WT vs. KO. In (*E*), the *P*-value represents treatment with carvedilol vs. without carvedilol for each group.

recycling of the WT β$_1$-AR in AKAP5$^{-/-}$ NMVM without affecting the trafficking of WT β$_2$-AR in these cells.[8] Therefore, we wondered whether CaN bound to AKAP5 was primarily responsible for this selectivity. We addressed this question by repeating the recycling experiments in cells that expressed the WT β$_1$-AR along with peptides that competed with endogenous CaN in binding to AKAP5 (*Figure 5C*). Plasmids harbouring the cDNA for a GFP-tagged peptide corresponding to the CaN-binding site between amino acids 321−360 of AKAP5 and another that expressed the core VIVIT sequence that binds avidly to CaN were used.[11,12] Binding to AKAP5 was verified by co-IP's, where by ectopic expression of AKAP5 (312−360)-GFP interfered with AKAP5 binding to CaN, while soluble GFP did not (*Figure 4D*). Expression of the empty GFP vector 'pseudo-green colour' did not interfere with the membranous localization, agonist-mediated internalization, or recycling of internal WT β$_1$-AR 'pseudo-red colour' (*Figure 5C*, f'−j'). However, recycling of the agonist-internalized β$_1$-AR was inhibited in cells that expressed the CaN-binding peptides (*Figure 5C*, k'−o' and p'−t'). These results along with the findings of Li *et al.*[8] indicate that CaN bound to AKAP5 was critical for recycling of cardiac β$_1$-AR.

3.6 Reversal of the AKAP5$^{-/-}$ cardiac phenotype with carvedilol but not with CsA

To find out if the compromised cardiac performance associated with loss of AKAP5 bears similarities to cardiac insufficiency and failure in humans, we determined the effect of carvedilol on these parameters in age-matched AKAP5$^{+/+}$ vs. AKAP5$^{-/-}$ mice. Carvedilol is a non-selective β-blocker as well as an α$_1$-AR blocker that is widely used in the management of heart failure in humans.[10] Carvedilol was administered to 4-month-old mice in chow at a concentration of 1500 p.p.m. for 10 days.[11] Echocardiography of WT AKAP5$^{+/+}$ mice before and after carvedilol indicated that this compound has no effect on haemodynamic parameters (*Figure 6*). Echocardiography of AKAP5$^{-/-}$ littermates, however, showed a significant improvement in cardiac performance in both male and female mice (*Figure 6*). Carvedilol increased the ejection fraction by 26 ± 4%, markedly reduced systolic LV volumes by 43 ± 8% in AKAP5$^{-/-}$ mice, and produced a 30 ± 6% increase in the stroke volume (*Figure 6A*). In aggregate, these results indicate that haemodynamic parameters in AKAP5$^{-/-}$ after carvedilol were not significantly different from those in untreated AKAK5$^{+/+}$ mice ($P > 0.05$, $n = 8$).

Since ablation of the AKAP5 locus resulted in significant activation of CaN, we evaluated whether inhibition of CaN with CsA would affect the cardiac phenotype of AKAP5$^{-/-}$ mice (*Table 1*). A 14-day regimen of subcutaneous administration of 10 mg/kg/day of CsA did not produce a statistically significant effect on cardiac haemodynamics either in AKAP5$^{+/+}$ or in AKAP5$^{-/-}$ mice.

Next, we determined if improvement in cardiac function of AKAP5$^{-/-}$ mice by carvedilol was mediated, in part, by modulating the activities of CaN and CaMKII. Four-month-old AKAP5$^{+/+}$-NFAT-Luc$^{Tg/?}$ mice and AKAP5$^{-/-}$-NFAT-Luc$^{Tg/?}$ mice were fed chow supplemented with carvedilol for 10 days (*Figure 4E*). Activities of luciferase in cardiac extract from carvedilol-fed AKAP5$^{+/+}$-NFAT-Luc$^{Tg/?}$ mice or AKAP5$^{-/-}$-NFAT-Luc$^{Tg/?}$ mice were 555 ± 50 and 919 ± 104 RLU/min/mg, respectively ($P > 0.05$).

The effect of carvedilol on CaMKII was determined in 4-month-old AKAP5$^{+/+}$ vs. AKAP5$^{-/-}$ mice that were fed chow or chow containing carvedilol for 10 days (*Figure 4F*). Activities of CaMKII in heart homogenates of AKAP5$^{+/+}$ mice on regular chow or chow with carvedilol were
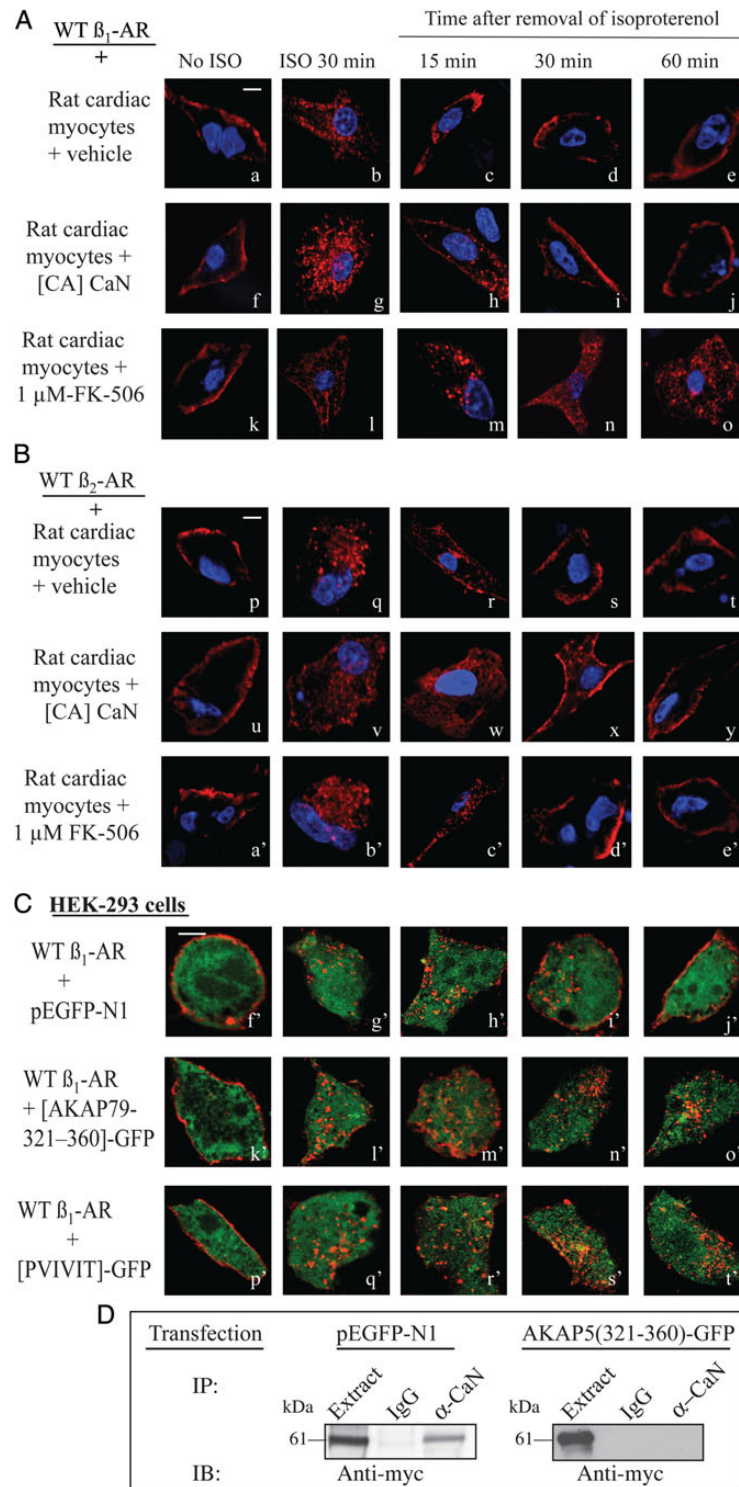
Figure 5 Effect of CaN on trafficking of the β_1-AR in cardiac myocytes. (*A* and *B*) Rat neonatal cardiac myocytes were infected with 100 m.o.i. with adenoviruses harbouring the FLAG-tagged β_1-AR (a−e and k−o) or FLAG-tagged β_2-AR (p−t and a′−e′). Another set of cells was infected with 50 m.o.i. of β_1-AR adenovirus and 50 m.o.i. of constitutively active (CA) CaN adenovirus (f−j and u−y). Cells infected with the β_1-AR or β_2-AR adenovirus were exposed to buffer or 1 µM FK-506 and then labelled Cy3-anti-FLAG IgG. Cells were washed, fixed (a, f, k, p, u, and a′), or exposed to 10 µM isoprenaline for 30 min and then fixed (b, g, l, q, v, and b′). The remaining slides were incubated with 100 µM alprenolol and subjected to recycling conditions for the indicated time period and then fixed. Shown is pseudo-colour 'red' for the β_1-AR and 'violet' for nuclei. (*C*) HEK-293 cells stably expressing FLAG β_1-AR were transiently transfected with the empty pEGFP-N1 (f′−j′), AKAP5 (321−360)-GFP (k′−o′), or with PVIVIT-GFP (p′−t′) vector then processed for trafficking as described above. Scale bars represent 5 µm. (*D*) Extracts from HEK-293 cells expressing AKAP5 and either pEGFP-N1 or AKAP5 (312−360)-GFP peptide were IP's on anti-AKAP5 or pre-immune resin. Then extracts (2% of total) or eluate were subjected to western blotting and probed with anti-myc antibody to detect α-CaN.
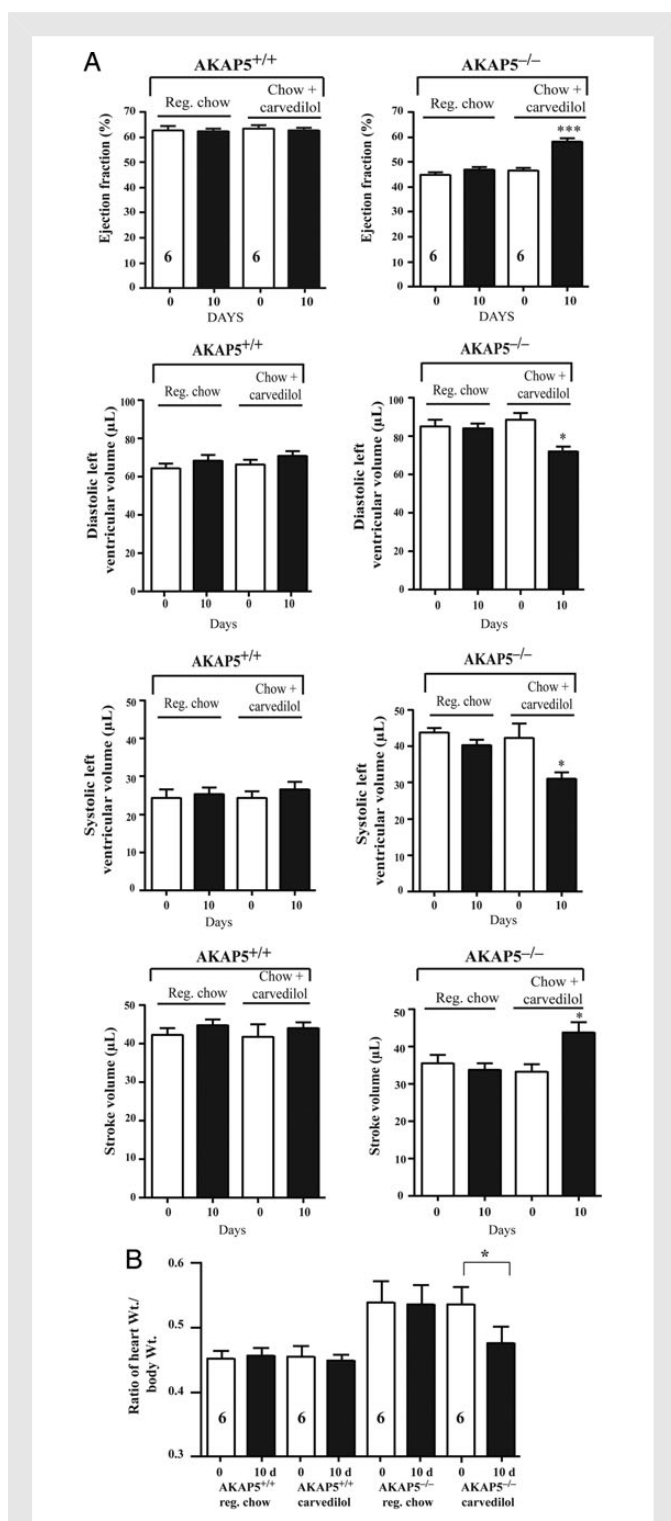
Figure 6 Effect of carvedilol on cardiac haemodynamics in AKAP5$^{+/+}$ vs. AKAP5$^{-/-}$ mice. (*A*) Four-month-old AKAP5$^{+/+}$ or AKAP5$^{-/-}$ mice were echoed. Then, fed control chow or chow with 1500 p.p.m. of carvedilol for 10 days and echoed again. (*B*). Ratios of heart-to-body weight. The data were plotted as means $\pm$ SE and then compared among different groups. *n*, represents the number of mice in each condition. In (*A*), *$P < 0.05$ and ***$P < 0.001$ represent the *P*-value of either base in WT vs. base in KO mice or carvedilol in WT vs. carvedilol in KO. In (*B*), the *P*-value represents either base in WT vs. base in KO or carvedilol in WT vs carvedilol in KO mice.

269 ± 36 and 283 ± 44 pmol/mg protein/min, respectively ($P > 0.05$). Activities of CaMKII in heart homogenates of AKAP5$^{-/-}$ mice on regular chow or chow containing carvedilol were 509 ± 58 and 364 ± 66 pmol/mg protein/min, respectively ($P > 0.05$). Therefore, the activity of CaMKII in carvedilol-fed AKAP5$^{-/-}$ mice was not significantly different from that of CaMKII in AKAP5$^{+/+}$ without or with carvedilol ($P > 0.05$). In addition, carvedilol normalized the activity of CaMKII (*Figure 4D*) and the ratio of heart-to-body weight (*Figure 6B*) in AKAP5$^{-/-}$ mice to its levels in AKAP5$^{+/+}$ mice. These results indicate that AKAP5 is cardioprotective by virtue of its inhibition of cardiac CaN and CaMKII signalling cascades and highlight a link between AKAP5 and cardiac β_1-AR in this phenomenon.

4. Discussion

4.1 Role of AKAPs in cardiac physiology and pathology

Several approaches have been used to investigate the role of the more than 14 cardiac AKAPs in regulating a variety of cardiac events. Knock-down of muscle AKAPβ with an siRNA in cardiac myocytes inhibited α- and β-adrenergic agonist-induced hypertrophy.[23] siRNA-mediated knockdown of cardiac Yotiao (AKAP9) indicated that this AKAP was involved in regulating the activity of cardiac potassium channels and β-adrenergic signalling.[24] The gene deletion approach was used in characterizing the physiological function of AKAP$_{lbc}$ (AKAP13), AKAP5, and AKAP15/18 (AKAP7). Ablation of AKAP13 was embryonically lethal, indicating that this AKAP was involved in genetic programming.[25] Deletion of the various isoforms of AKAP7 did not affect the responsiveness of cardiomyocytes to adrenergic stimulation.[26] Global deletion of AKAP5 interfered with β-agonist-mediated regulation of $[Ca^{2+}]_i$ and phosphorylation of substrates involved in Ca^{2+} handling in cardiomyocytes derived from adult AKAP5$^{-/-}$ mice.[7] However, isoprenaline elicited $[Ca^{2+}]_i$ were maintained in cardiomyocytes derived from AKAP5 D36 mice, which express an AKAP5 construct that lacks the PKA-binding site.[7] Another study indicated that β_1-AR-mediated increase in cyclic AMP was $\sim$40% higher in AKAP5$^{-/-}$ cardiac myocytes than in AKAPP5$^{+/+}$ myocytes.[8] These results imply that AKAP domains other than the PKA-binding domain were involved in cardiac effects of AKAPs.

4.2 Roles of enhanced signalling by the β_1-AR or defective trafficking of cardiac β_1-AR in genesis of the cardiac phenotype of AKAP5$^{-/-}$ mice

Ablation of AKAP5 might lead to cardiac hypertrophy and insufficiency by two independent mechanisms. First, myocardial AKAP5 interacts with AC isoforms V and VI and facilitates their phosphorylation by AKAP5-anchored PKA to inhibit cyclic AMP formation.[2,3] AKAP5-anchored PKA is selectively activated by the β-AR signalling pathway to blunt excessive signalling by the receptor.[2,3] In this view, ablation of AKAP5 in NMVM augmented β_1-AR signalling to cyclic AMP by alleviating the inhibitory effect of AKAP5-anchored PKA on AC-V and VI. Enhanced β_1-AR signalling to cyclic AMP and subsequently to downstream hypertrophic pathways such as Akt, exchange protein directly activated by cyclic AMP (Epac), CaMKII and many other kinases might explain the cardiac phenotype observed *in vitro* in NMVM and *in vivo* in the heart (*Figure 1*).[17,22,27,28] Secondly, ablation of AKAP5 interfered

Table 1 Effect of CsA on echocardiographic parameters in WT vs. AKAP5 KO littermates

	Solvent AKAP5$^{+/+}$		Cyclosporine A AKAP5$^{+/+}$		Solvent AKAP5$^{-/-}$		Cyclosporine A AKAP5$^{-/-}$	
	Before (*n* = 8)	After	Before (*n* = 8)	After	Before (*n* = 8)	After	Before (*n* = 8)	After
EF (%)	63.1 + 4.6	63.6 + 3.6	62.5 + 2.9	60.6 + 3.1	46.9 + 2.5	46.6 + 3.4	46.7 + 2.7	50.5 + 3.6
FS (%)	43.8 + 2.2	44.0 + 2.4	43.4 + 2.3	42.8 + 2.4	33.4 + 1.5	33.0 + 2.7	33.7 + 3.3	35.5 + 3.0
LV Vol$_d$ (μL)	64.1 + 4.6	65.0 + 5.3	65.9 + 4.3	59.7 + 5.7	79.2 + 6.3	79.3 + 6.9	78.4 + 6.1	76.4 + 6.3
LV Vol$_s$ (μL)	23.7 + 3.4	23.8 + 3.7	24.9 + 2.8	22.7 + 3.2	42.9 + 4.6	42.5 + 3.5	41.6 + 5.5	38.2 + 4.4
SV (μL)	40.4 + 4.1	41.3 + 4.3	41.0 + 4.0	36.9 + 4.4	37.2 + 4.8	36.8 + 4.3	36.7 + 3.8	38.2 + 4.2
CO/BW	0.74	0.75	1.02	0.93	0.77	0.71	0.69	0.74

Echocardiography was before and after the administration of 10 mg CsA /kg/day or solvent for 14 days to 4-month-old mice.
EF, ejection fraction; FS, Fractional sgortening; LV Vold, diastolic left ventricular volume; LV Vols, systolic left ventricular volume; SV, stroke volume; CO/BW, ratio of cardiac output/min/body weight in grams.

with recycling of the agonist-internalized β$_1$-AR in NMVM.[8] We believe that altered trafficking of the myocardial β$_1$-AR and the role of CaN bound to AKAP5 in this phenomenon also plays a significant role in hypertrophy of AKAP5$^{-/-}$ mice. There is ample evidence that internalized β$_2$-AR and other GPCR still signal intracellularly.[29,30] Thus, retention of the agonist-internalized β$_1$-AR might prolong the duration of G$_s$-signalling in the heart of AKAP5$^{-/-}$ mice. Moreover, internalization was required for β$_1$-AR-induced cardiac hypertrophy.[31] Consequently, enhanced hypertrophic signalling either from membrane-bound or internalized β$_1$-AR could potentially contribute to hypertrophy in hearts of AKAP5$^{-/-}$ mice.

4.3 Differential resistance of AKAP5$^{-/-}$ mice to isoprenaline

Indiscriminate inhibition of PKA by global expression of a PKA inhibitory peptide or by delocalization of PKA from AKAP by the Ht-31 peptide blunted signalling by the β-AR.[15,32,33] Similarly, isoprenaline-mediated increase in [Ca^{2+}]$_i$ was markedly reduced in AKAP5$^{-/-}$ cardiomyocytes.[7] Nichols et al.[7] attributed uncoupling between excitation and contraction in response to isoprenaline in AKAP5$^{-/-}$ cardiomyocytes to loss of PKA-dependent phosphorylation of ryanodine2 receptors, phospholamban, and LTCC that were associated with caveolin 3. We also determined that chronic isoprenaline infusions failed to further diminish cardiac haemodynamic in AKAP5$^{-/-}$ mice (*Figure 3*), presumably by similar mechanisms to those alluded to by Nichols et al.[7]

4.4 Role of PKA-independent up-regulation of downstream signalling by Ca^{2+}/CaM in genesis of the cardiac phenotype of AKAP5$^{-/-}$ mice

Ablation of AKAP5 did not alter the expression of the immunoreactive catalytic αA subunit of CaN,[7] so what is the cause of the marked elevation of CaN activity in AKAP5$^{-/-}$ mice? We speculate that loss of AKAP5 was involved in activating CaN because expression of AKAP5 markedly reduced the activity of CaN and myocardial hypertrophy after isoprenaline infusion or pressure overload.[11,34,35] A role for enhanced CaN activity in cardiac hypertrophy is well established, particularly that inhibition of CaN with CsA (15 mg/kg/days) or FK506 has been shown to prevent cardiac hypertrophy in transgenic

mice.[36,37] However, in our hands, these doses resulted in unacceptable side effects, such as chewing of extremities and early death in WT and in AKAP5$^{-/-}$ mice. Assuming that our dose of CsA was sufficient, we interpret the inability of CsA to reverse the AKAP5$^{-/-}$ phenotype as an indication for the preponderance of other signalling or transcriptional pathways in imparting the AKAP5$^{-/-}$ cardiac phenotype. One such other pathway is the cardiac CaMKII pathway, which is activated by Ca^{2+} and by the β$_1$-AR via PKA-independent mechanisms involving altered Epac, β-arrestin signalling, and other kinases and phosphatases.[17,22,27,28,38]

4.5 Reversal of the AKAP5$^{-/-}$ phenotype with carvedilol

The effects of AKAP5 in the heart were mediated by several mechanisms. Therefore, we used carvedilol, a third generation β-blocker, that acts by multiple mechanisms to ameliorate heart failure in humans.[10] These mechanisms involve blockade of α$_1$- and β-AR, inhibition of reactive oxygen species, and biased agonism involving the activation of G protein receptor kinase/β-arrestin signalling.[10,39] The mechanism underlying the beneficial effects of carvedilol in AKAP5$^{-/-}$ mice is not known, but might involve its ability to inhibit aberrant β$_1$-AR, G protein-dependent (G$_s$-mediated), G protein-independent (Epac/β-arrestin), and numerous other kinases/phosphatases and their downstream signalling effectors in the heart.[15,22,27−31,39] Our results indicate that carvedilol normalized the activities of CaN and CaMKII in AKAP5$^{-/-}$ mice to their baseline levels in WT mice.

This study indicates that hypertrophy and insufficiency in AKAP5$^{-/-}$ mice bear similarities to these conditions in humans in that they are progressive and mediated by aberrant GPCR and Ca^{2+} signalling. These results emphasize an unexpected cardioprotective role for AKAP5, which is exerted on β$_1$-AR, CaN, and CaMKII signalling that is worthy of further translational investigation.

Conflict of interest: none declared.

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