

A background Ca^{2+} entry pathway mediated by TRPC1/TRPC4 is critical for development of pathological cardiac remodelling

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Aims

Pathological cardiac hypertrophy is a major predictor for the development of cardiac diseases. It is associated with chronic neurohumoral stimulation and with altered cardiac Ca^{2+} signalling in cardiomyocytes. TRPC proteins form agonist-induced cation channels, but their functional role for Ca^{2+} homeostasis in cardiomyocytes during fast cytosolic Ca^{2+} cycling and neurohumoral stimulation leading to hypertrophy is unknown.

Methods and results

In a systematic analysis of multiple knockout mice using fluorescence imaging of electrically paced adult ventricular cardiomyocytes and Mn^{2+} -quench microfluorimetry, we identified a background Ca^{2+} entry (BGCE) pathway that critically depends on TRPC1/C4 proteins but not others such as TRPC3/C6. Reduction of BGCE in TRPC1/C4-deficient cardiomyocytes lowers diastolic and systolic Ca^{2+} concentrations both, under basal conditions and under neurohumoral stimulation without affecting cardiac contractility measured in isolated hearts and *in vivo*. Neurohumoral-induced cardiac hypertrophy as well as the expression of foetal genes (ANP, BNP) and genes regulated by Ca^{2+} -dependent signalling (RCAN1-4, myomaxin) was reduced in TRPC1/C4 knockout (DKO), but not in TRPC1- or TRPC4-single knockout mice. Pressure overload-induced hypertrophy and interstitial fibrosis were both ameliorated in TRPC1/C4-DKO mice, whereas they did not show alterations in other cardiovascular parameters contributing to systemic neurohumoral-induced hypertrophy such as renin secretion and blood pressure.

Conclusions

The constitutively active TRPC1/C4-dependent BGCE fine-tunes Ca^{2+} cycling in beating adult cardiomyocytes. TRPC1/C4-gene inactivation protects against development of maladaptive cardiac remodelling without altering cardiac or extracardiac functions contributing to this pathogenesis.

Keywords

Calcium • Ion channels • Cardiac remodelling • Background Ca^{2+} entry • TRPC1/TRPC4

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Translational perspective

Pathological cardiac hypertrophy is a major predictor for the development of cardiac diseases. Neuroendocrine factors such as norepinephrine and angiotensin II trigger Ca^{2+} -dependent processes leading to cell growth and cardiac hypertrophy. Here, we report the identification of a constitutively active background Ca^{2+} entry (BGCE) pathway that is not affected by inhibition of voltage-gated Ca^{2+} channels but critically depends on the presence of TRPC1 and TRPC4 proteins, members of the TRP family of cation channels. It fine-tunes Ca^{2+} cycling in beating cardiomyocytes under basal conditions and during neurohumoral stimulation. Suppression of BGCE protects against development of maladaptive cardiac remodelling without evidence for alterations in cardiac or extra-cardiac functions and may represent a potential new therapeutic strategy to attenuate the pathogenesis of associated diseases.

Introduction

Pathological cardiac hypertrophy is a major predictor for the development of cardiac diseases.¹ In cardiomyocytes, neurohumoral stimuli such as catecholamines or angiotensin II (AngII) activate G-protein-dependent signalling pathways that stimulate Ca^{2+} dependent processes leading to myocyte growth and cardiac hypertrophy.² Beating cardiomyocytes display fast cytosolic Ca^{2+} cycling originating from a spatiotemporally coordinated interplay of voltage-gated Ca^{2+} channels, Na^+ – Ca^{2+} exchangers, ryanodine receptors, and plasma membrane and SERCA pumps.³ It is unclear to which extent cardiac remodelling processes associated with cardiac hypertrophy are caused solely by modulating channels and pumps involved in fast Ca^{2+} cycling or whether alternative Ca^{2+} entry pathways may fine-tune cardiac Ca^{2+} homeostasis persistently and contribute to cardiac hypertrophy.⁴ A longer lasting modulation of diastolic Ca^{2+} levels has been suggested as a regulatory mechanism for the development of cardiac hypertrophy, which can result from alterations of sarcoplasmic reticulum (SR) Ca^{2+} release and/or by altered expression of proteins involved in plasma membrane Ca^{2+} transport.⁴ The complexity of Ca^{2+} -dependent regulation of cardiac hypertrophy *in vivo* becomes evident as a large increase or reduction of voltage-gated L-type Ca^{2+} currents both result in mild cardiac hypertrophy.^{5,6}

TRPC proteins form ion channels that contribute to Ca^{2+} entry directly or indirectly through activation of voltage-gated Ca^{2+} channels.⁷ The effects of over-expressing TRPC transgenes or dominant negative variants and knockdown of TRPC genes suggested that members of the TRPC subfamily may be involved in cardiac Ca^{2+} signalling and hypertrophy development.^{8–12} However, their impact on the diastolic level and amplitude of fast cytosolic Ca^{2+} cycling in beating adult cardiomyocytes and during neurohumoral stimulation triggering hypertrophy is unknown. Nevertheless, it was proposed that substances antagonizing TRPC channels may work as drugs to suppress cardiac hypertrophy. To develop such antagonists, it has to be considered, which specific members of the TRPC subfamily are essential constituents of channels involved in cardiac remodelling processes. Additionally, it is still unknown whether these proteins affect other cardiovascular functions and whether extra-cardiac TRPC channels alter the development of cardiac hypertrophy. Here, we identify TRPC1 and TRPC4 as constituents of a novel background Ca^{2+} entry (BGCE) pathway in adult cardiomyocytes, which determines diastolic and systolic Ca^{2+} concentrations in beating cells. TRPC1 and TRPC4 together are required for

maladaptive cardiac remodelling without affecting cardiac contractility and rhythmicity or systemic blood pressure.

Methods

An expanded Materials and methods is available at Supplementary material online.

Animal experiments

All animal experiments were reviewed and approved in accordance with the ethic regulations and the animal welfare committees of the Universities of Saarland and Heidelberg. Isoproterenol (Iso, 30 mg/kg/day) and AngII (3 mg/kg/day) were infused via Alzet pumps over 7 and 14 days, respectively; pumps filled with saline (NaCl 0.9%) were used as control. Blood pressure and heart rate were measured with telemetric transmitters (PA-C10, Data Science International). Ivabradine was administered via chow pellets. Transverse aortic constriction (TAC, 5 weeks) was performed by tying a suture ligature against a 27-gauge needle and control mice underwent a sham operation. Plasma angiotensinogen and AngII concentrations were determined by Angiotensinogen (JP27413, IBL, Germany) and AngII (Uscn, Life Science Inc.) ELISA assays. Renin activity from the perfusate of isolated kidneys was determined from quantification of AngI by radioimmunoassay (Byk and DiaSorin). To measure cardiac function, a conductance catheter (SPR-835, Millar) inserted into the left ventricular from hearts mounted in a working heart apparatus (Hugo Sachs Elektronik-Harvard) was used. Cardiac dimensions and contractility were assessed by echocardiography under anaesthesia (1.5% Isoflurane) using a Vevo 770 system (Visualsonics).

Fluorescence imaging of adult mouse cardiomyocytes

Electrically evoked Ca^{2+} transients were measured in Fura2-loaded ventricular adult mouse myocyte using a 'post-rest' protocol. Sarcoplasmic reticulum- Ca^{2+} content was analysed in cardiomyocytes from caffeine-induced Ca^{2+} transients. Ca^{2+} sparks were studied in Fluo4-loaded myocytes at imaging frequencies of 100 Hz. Mn^{2+} -induced Fura2-fluorescence quenching was employed to measure Ca^{2+} entry using nominally Ca^{2+} -free solutions. Iso and AngII stimulation of adult mouse ventricular myocytes was performed after reaching steady-state conditions by field stimulation (5 min, 1 Hz, 12 V, alternating polarities).

RNA isolation and expression analysis

Total heart RNA was isolated with peqGOLD-RNAPure reagent (peq-Lab) and purified by extraction steps with diethyl ether. Quantity, purity, and integrity of the RNA samples were controlled by spectrophotometry (NanoQuant, Tecan) and microfluidic analysis (Bioanalyzer 2100,

Agilent Technologies). For real-time quantitative PCR, 10 ng cDNA were used for TaqMan Gene Expression Assay. Quantitative PCR was performed with a Universal Probe Library (Roche) for Rcan1-4 and Myomaxin, and the specific TaqMan probe for mouse GAPDH (Applied Biosystems, Product Nr. Mm03302249) and detection on a 7500-Fast Cyclor (Applied Biosystems). For expression analysis by NCounter NanoString technology RNA was hybridized with a Nanostring Gene Expression CodeSet and analysed using the nCounter Digital Analyzer. Corresponding counts (normalized counts) are presented relative to expression levels of control genes (Actb, Hprt1, Tbp, Ubc, and Gapdh).

Histopathological analysis

Images were acquired with a digital colour camera (MRc5, Zeiss) and were analysed with the AxioVision software (Zeiss). To quantify the cross-sectional area >100 cells/mouse were analysed through the left ventricle free wall. Quantification of interstitial cardiac fibrosis was performed with the AutoMeasure module of AxioVision software 4.8.1 (Zeiss).

Data analysis

Analyses were made with Origin v8.1.13.88 (OriginLab Corporation, USA) or Prism 5.0c (GraphPad Software Inc.). Data are displayed as bar graphs or scatter plots with indication of mean \pm SD. All analyses

were made using each mouse as experimental unit. Statistical analyses were performed using the appropriate tests (unpaired or paired t-test, one- or two-way ANOVA, Mann–Whitney or Kruskal–Wallis) depending on the result of normality tests (Shapiro–Wilk or D'Agostino–Pearson omnibus) and Levene's test for equal variance. Differences with $P < 0.05$ were considered as statistically significant. Significances were depicted as actual P -values or as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

TRPC1 and TRPC4 form a background Ca^{2+} entry pathway that determines diastolic and systolic $[\text{Ca}^{2+}]_i$ and responses to neurohumoral stimulation

We studied putative effects of TRPC1/C4 and TRPC3/C6 double knockout (DKO) on changes of intracellular Ca^{2+} in electrically paced adult ventricular myocytes. Resting and diastolic Ca^{2+} concentrations and the amplitude of Ca^{2+} transients were reduced in TRPC1/C4-DKO but not in TRPC3/C6-DKO (Figure 1A and B) whereas the post-rest behaviour of global calcium transients (Figure 1A) and the current density of voltage-gated L-type Ca^{2+}

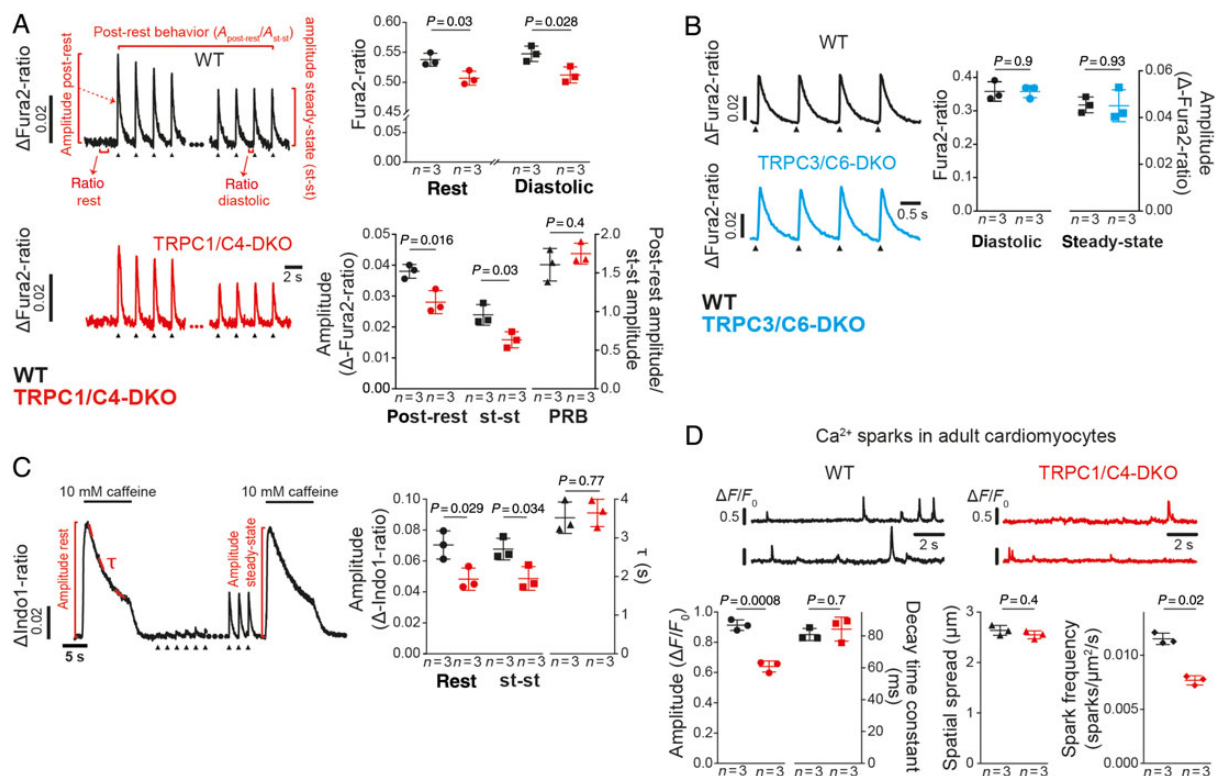


Figure 1 Ca^{2+} signalling in cardiomyocytes from TRPC1/C4-DKO is reduced. (A) Representative electrically evoked Ca^{2+} transients (0.5 Hz, arrow heads) in adult ventricular myocytes. Here resting Ca^{2+} (rest) and diastolic Ca^{2+} levels during steady-state (st-st) pacing, and amplitudes of Ca^{2+} transients post-rest and during steady-state pacing are depicted. The post-rest behaviour, PRB, (post-rest amplitude/st-st amplitude) is shown (44–55 cells/group). (B) Analysis of electrically evoked Ca^{2+} transients (1 Hz) in TRPC3/C6-DKO myocytes (119–191 cells/group). (C) Analysis of caffeine-induced Ca^{2+} transients in resting cardiomyocytes or immediately after a 3-min pacing period (st-st conditions). Typical transients are depicted highlighting the amplitudes (65–75 cells/group) and decay time constant (τ) (52–68 cells/group). (D) Recordings of two different spark sites and the corresponding analysis of decay time constant, spatial spread and frequency (24–32 cells/group).

channels (see Supplementary material online, *Figure S1A*) were unaltered in TRPC1/C4-DKO.

Sarcoplasmic reticulum- Ca^{2+} content, estimated from the amplitude of caffeine-induced Ca^{2+} transients, was reduced under resting and steady-state conditions; however, Ca^{2+} extrusion, which is mainly mediated by NCX activity in mice¹³ and can be correlated with the decay time constant of the caffeine-induced transients (*Figure 1C*), was unchanged in TRPC1/C4-DKO mice as was the decay of electrically evoked Ca^{2+} transients, which correlates with SERCA activity (see Supplementary material online, *Figure S1B*). Analysis of Ca^{2+} sparks revealed that the amplitude and frequency of Ca^{2+} , but not the decay time constant and spatial spread, were reduced in cells from TRPC1/C4-DKO (*Figure 1D*).

To investigate Ca^{2+} entry across the plasma membrane, we used Mn^{2+} -quench microfluorimetry. *Figure 2A* shows a typical Fura2-fluorescence trace with the rate of quenching (α) being proportional to the magnitude of Mn^{2+} entry. The basal Mn^{2+} quench rate and the Gd^{3+} -sensitive portion of this Mn^{2+} -entry were reduced in TRPC1/C4-DKO cells (*Figure 2A*). This Mn^{2+} -entry was not affected by voltage-gated calcium channel blockers (see Supplementary material online, *Figure S1C*) or deletion of TRPC3/C6 proteins (*Figure 2B*). Importantly, we found a significantly larger Mn^{2+} entry

in WT cardiomyocytes after chronic neurohormonal stimulation with Iso or AngII but this increase was abrogated in TRPC1/C4-DKO cells (*Figure 2C*).

Next, we addressed the question whether and how the lack of TRPC1/C4 might modulate Iso- and AngII-evoked changes of Ca^{2+} transients. In cardiomyocytes from WT and TRPC1/C4-DKO mice application of Iso resulted in an equal relative increase in the amplitude of Ca^{2+} transients, whereas peak Fura2-ratios were substantially reduced in TRPC1/C4-DKO mice (*Figure 3A*; see Supplementary material online, *Figure S2A*). Interestingly, both, peak Fura2-ratios and their relative increases during AngII-application, were significantly reduced (*Figure 3B*; see Supplementary material online, *Figure S2B*). In cardiomyocytes from TRPC3/C6-DKO mice, Ca^{2+} responses to Iso or AngII stimulation were not altered (*Figure 3C* and *D*; see Supplementary material online, *Figure S2C* and *D*).

TRPC1/C4-DKO are protected from development of maladaptive cardiac hypertrophy

To identify the contribution of BGCE and TRPCs (see Supplementary material online, *Figure S3*) to the development of cardiac

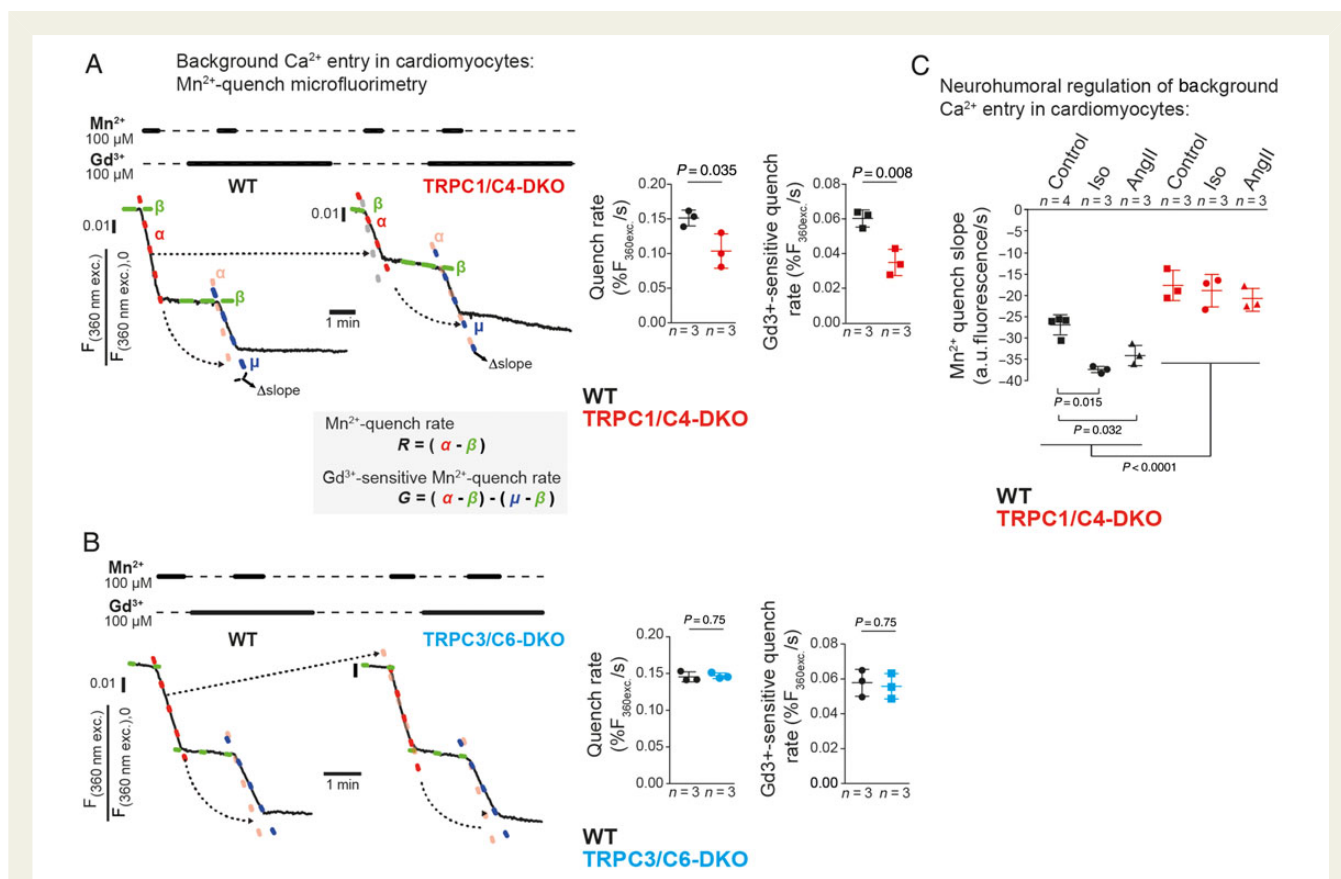
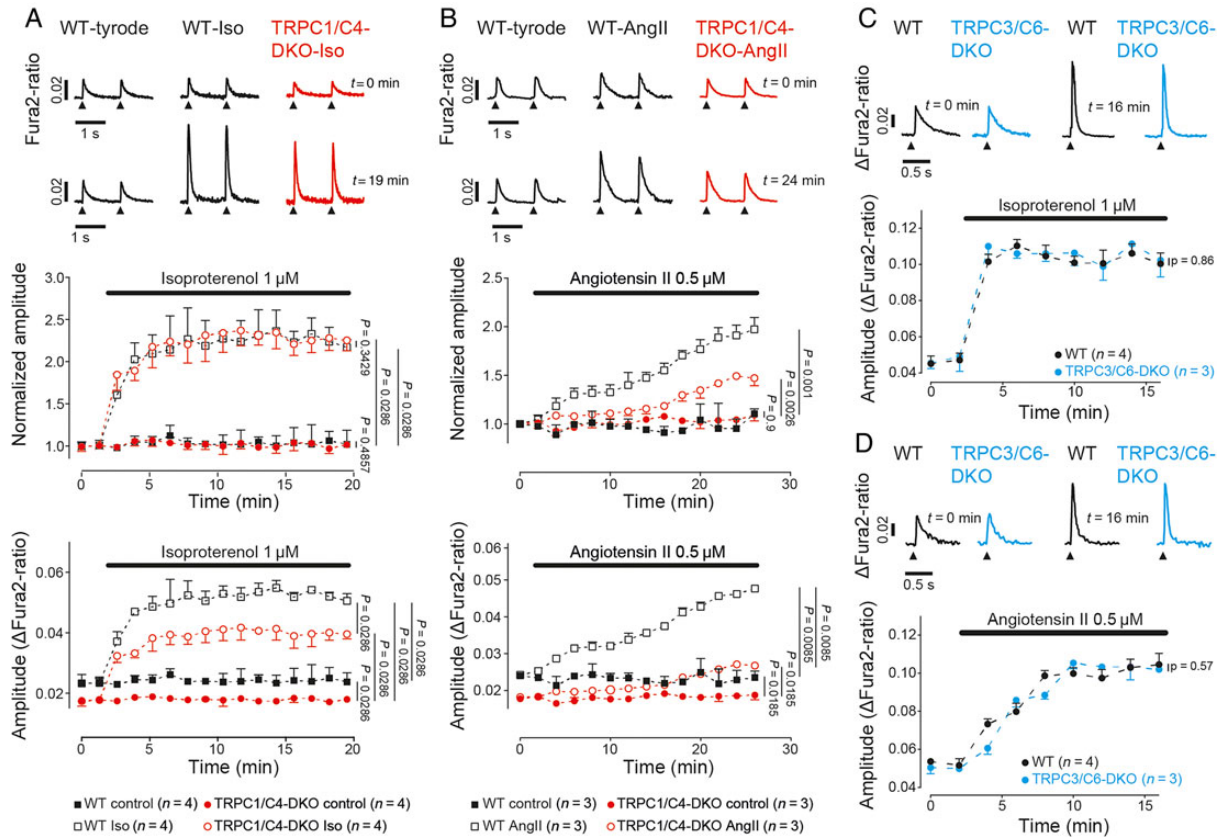


Figure 2 TRPC1/C4 form a background Ca^{2+} entry pathway in adult cardiomyocytes. (A) For quantifying constitutively active background Ca^{2+} entry pathways, we linearly approximated Mn^{2+} -quench rates (α or μ) and corrected them for bleaching of Fura2 (β). Quenching rates in the absence (R) and presence of Gd^{3+} (G , as ' Δ slope') were analysed (24–32 cells/group). (B) Mn^{2+} -quench in WT and TRPC3/C6-DKO cardiomyocytes (24–26 cells per group) as described in (A). (C) Mn^{2+} -quench (1 mM, 37°C) in cells from mice treated with Iso or AngII (174–471 cells/group).



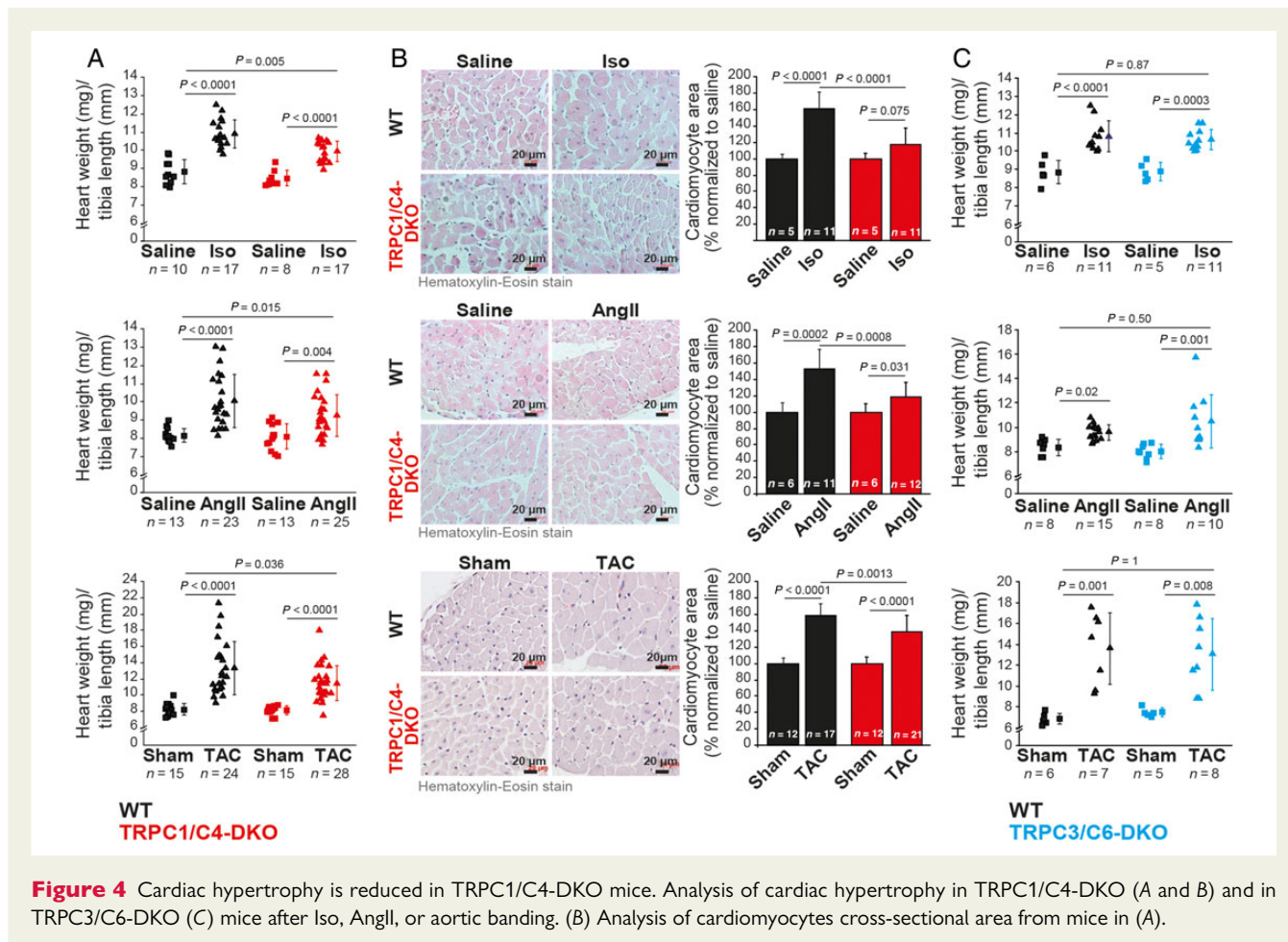
hypertrophy, we analysed responses of TRPC1/C4-DKO mice to systemic infusion of Iso or AngII, or to increased afterload (TAC). In TRPC1/C4-DKO mice, cardiac hypertrophy was significantly reduced in all three conditions (Figure 4A and B). TRPC1 and TRPC4 are thought to interact with TRPC5,¹⁴ which appears to be up-regulated in patients with cardiac failure.¹⁵ We therefore analysed TRPC1/C4/5 triple KO mice and found no additional reduction in Iso-induced hypertrophy compared with TRPC1/C4-DKO mice (see Supplementary material online, Figure S4). However, mice lacking either TRPC1 or TRPC4 showed no reduced Iso- or AngII-induced hypertrophy responses (see Supplementary material online, Figure S5). Because cardiac-specific over-expression of TRPC6 and TRPC3 resulted in cardiac hypertrophy and failure,^{11,12} we tested whether both proteins have a causative role in these processes. We analysed TRPC3/C6-DKO mice to avoid compensatory effects of these TRPCs,¹⁶ but cardiac hypertrophy and fibrosis were not reduced (Figure 4C; see Supplementary material online, Figure S6).

In adverse cardiac remodelling, myocardial hypertrophy is accompanied by reduction of cardiac function, interstitial fibrosis, and the activation of a foetal gene program. In TRPC1/C4-DKO mice, both

the TAC-evoked reduction in ejection fraction (WT: -28.6% , DKO: -14.1% ; Figure 5A and Table 1) and the interstitial fibrosis (Figure 5B) were reduced by $\sim 50\%$. Accordingly, AngII-induced increases in *col1a1*, *col3a1*, and *col4a1* (Figure 5C) and in foetal gene expression, i.e. *Nppa* and *Nppb* (Figure 5D), were diminished. To characterize Ca^{2+} -dependent downstream signalling pathways involved in adverse cardiac remodelling, we quantified the expression RCAN1-4 as an endogenous calcineurin reporter¹⁷ and myomaxin as a transcriptional target of MEF2a.¹⁸ Expression of both was reduced in TRPC1/C4-DKO mice (Figure 5E).

Systemic pathways possibly contributing to the reduced cardiac hypertrophy in TRPC1/C4-DKO mice

Systemic administration of Iso or AngII activates signalling pathways in multiple organ systems that could influence the development of cardiac hypertrophy. However, Iso-induced renin release and its suppression by AngII were unaffected in TRPC1/C4-DKO (Figure 6A), as well as plasma AngII and angiotensinogen concentrations (Figure 6B). Likewise, Iso infusion resulted in a transient



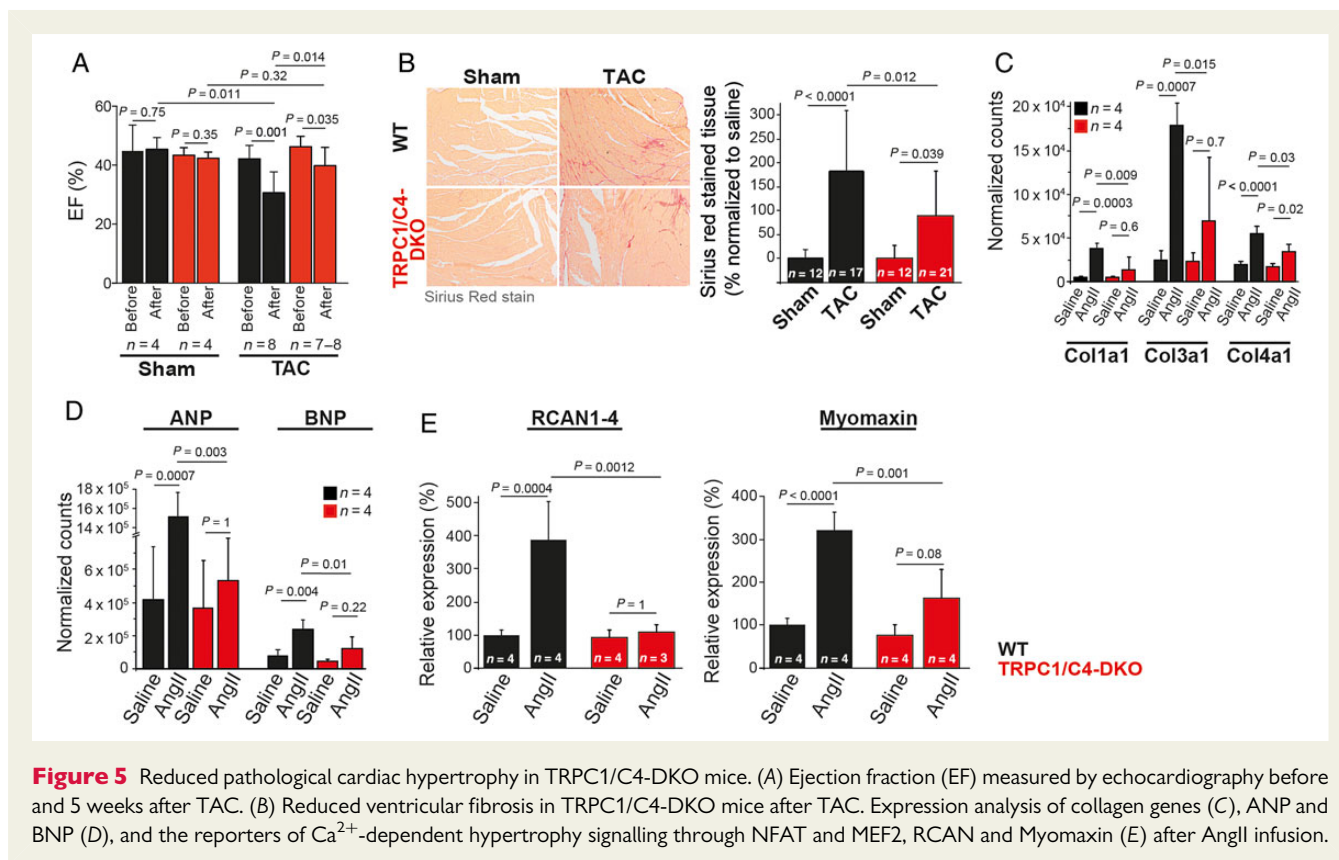
increase in mean arterial blood pressure (MAP), but it was indistinguishable from pre-implantation levels at Day 7 in both genotypes, while hypertrophic responses were still reduced (Figure 6C, inset). In contrast, AngII infusion in WT mice resulted in an ~ 45 mmHg increase in MAP (145.4 ± 10.2 mmHg; $n = 5$, $P = 0.0006$), but hypertrophy development was smaller compared with Iso treatment (see Supplementary material online, Figure S7A) supporting our notion that reduced hypertrophy in TRPC1/C4-DKO mice was not dependent on altered MAP responses. Intra-ventricular pressure measurements performed at the end of Iso treatment revealed no alterations in contractility in TRPC1/C4-DKO mice (see Supplementary material online, Table S1). Comparable results were acquired from working heart experiments using untreated mice except of a 12% increase in time constant of relaxation (Table 2). Nevertheless, parameters of the end-diastolic pressure–volume relationship were not altered in untreated TRPC1/C4-DKO mice and a detailed echocardiographic analysis revealed normal systolic and diastolic functions (Table 1).

Because elevations in the beating frequency can increase diastolic Ca^{2+} concentrations and induce hypertrophy¹⁹ and Iso-induced chronotropic responses were reduced in TRPC1/C4-DKO mice (Figure 6D), we evaluated whether this impaired response may contribute to the protection of hypertrophy development in TRPC1/C4-DKO mice. We used Ivabradine to decrease heart rates before

(Figure 6E) and during Iso infusion (Figure 6E and F); however, Ivabradine did not reduce Iso-induced hypertrophy (Figure 6E and F).

Discussion

Here, we identified a novel TRPC1/C4-dependent constitutively active BGCE pathway in cardiomyocytes that fine-tunes Ca^{2+} cycling and determines diastolic and systolic Ca^{2+} concentrations during basal electrical pacing and following neurohumoral stimulation. Likewise, TRPC1/C4-DKO mice develop reduced cardiac hypertrophy. Although other cardiac cell types could contribute to this phenotype, the decreased hypertrophic responses in TRPC1/C4-DKO mice can be explained by a reduced activity of the TRPC1/C4-dependent BGCE in adult ventricular myocytes which is not dependent on voltage-gated Ca^{2+} channels but is increased during neurohumoral-induced cardiac hypertrophy. Moreover, changes of diastolic and peak Ca^{2+} levels in TRPC1/C4-DKO cardiomyocytes were not brought about by alternative mechanisms, including decreased L-type Ca^{2+} current,³ SERCA pump activity or by increased Ca^{2+} extrusion from the cytosol by NCX or plasma membrane Ca^{2+} pump(s). Nevertheless, the SR- Ca^{2+} content was reduced resulting in lower amplitudes and frequencies of spontaneous Ca^{2+} sparks. Their decay time and spatial spread were

**Table 1** Cardiac function analysed by echocardiography

Parameters	WT	TRPC1/C4-DKO	WT		TRPC1/C4-DKO		P-value TAC vs. TAC
	Basal (n = 12)	Basal (n = 12)	Sham (n = 4)	TAC (n = 8)	Sham (n = 4)	TAC (n = 7)	
Body weight (g)	30.8 ± 1.5	30.4 ± 2.8	28.5 ± 5.2	31.6 ± 1.8	31.2 ± 0.96	31.1 ± 2.8	
P-value		0.683		0.146		0.935	0.690
HR (BPM)	425 ± 32	382 ± 34	400 ± 36	481 ± 35	368 ± 32	507 ± 53	
P-value		0.015		0.004		0.0012	0.280
LV volume diastole (μL)	96.1 ± 9.9	88.7 ± 9.9	101.3 ± 6.7	112.9 ± 18	95.0 ± 7.9	89.8 ± 13.1	
P-value		0.161		0.326		0.491	0.016
LV volume systole (μL)	55.1 ± 11.3	47.9 ± 7.4	53.1 ± 4.1	77.7 ± 21	54.8 ± 5.6	55.2 ± 13.1	
P-value		0.095		0.089		0.961	0.030
EF (%)	43.0 ± 5.5	46.2 ± 3.4	45.4 ± 4.0	30.6 ± 7.0	42.3 ± 2.0	39.8 ± 6.2	
P-value		0.303		0.008		0.468	0.019
MVE-wave (mm/s)	993 ± 142	930 ± 157	859 ± 35	1099 ± 142	913 ± 99	1283 ± 227	
P-value		0.330		0.021		0.014	0.078
MVA-wave (mm/s)	643 ± 49	620 ± 137	577 ± 46	448 ± 296	542 ± 62	333 ± 244	
P-value		0.593		0.498		0.149	0.596
IVRT (ms)	15.0 ± 2.2	16.0 ± 2.1	14.7 ± 2.3	11.1 ± 1.9	16.2 ± 1.6	9.9 ± 2.9	
P-value		0.318		0.014		0.003	0.358
MV E/A	1.55 ± 0.28	1.52 ± 0.18	1.46 ± 0.15	3.58 ± 3.9	1.69 ± 0.23	5.75 ± 3.7	
P-value		0.739		0.329		0.073	0.457

LV, left ventricle; EF, ejection fraction; IVRT, isovolumetric relaxation time; MVE, mitral valve early wave peak; MVA, mitral valve atrial wave peak.

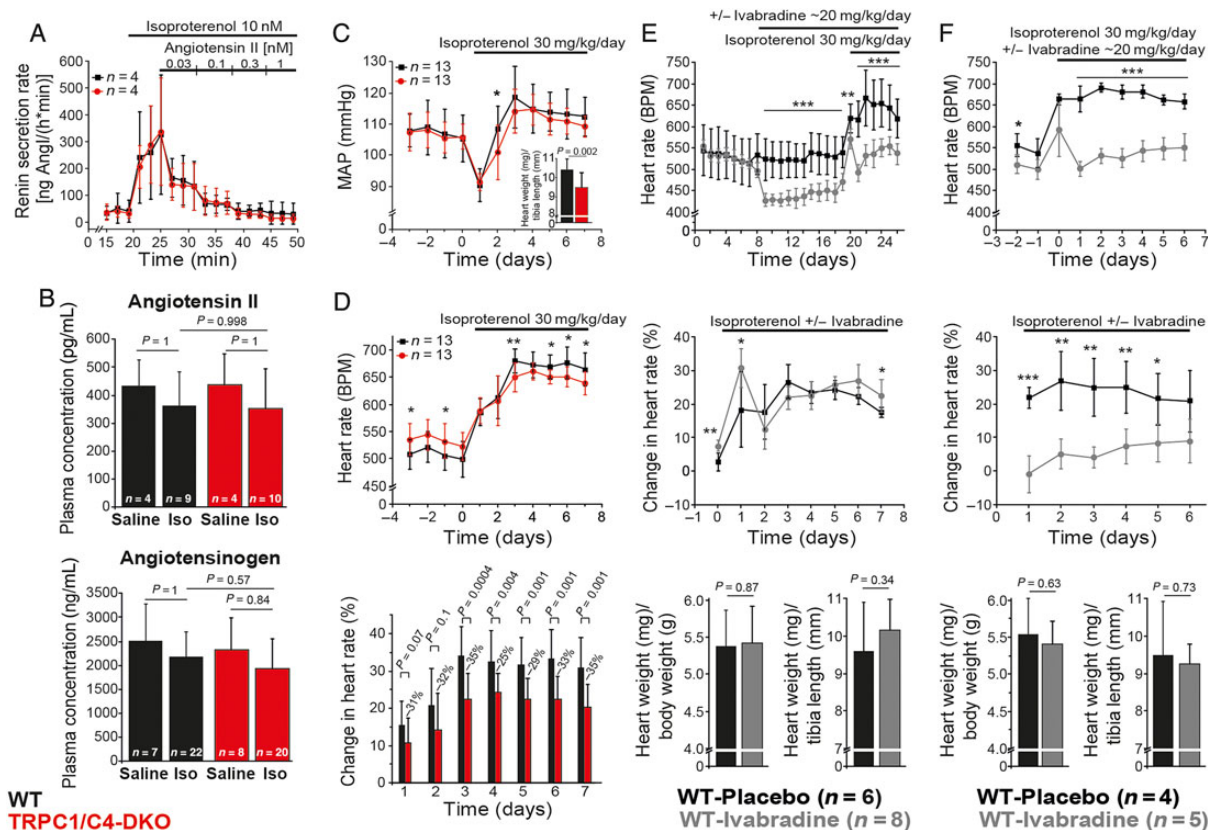


Figure 6 Systemic pathways contributing to the development of cardiac hypertrophy in TRPC1/C4-DKO. (A) Renin secretion in isolated perfused kidneys. (B) AngII and angiotensinogen concentration after Iso infusion. (C) Mean arterial blood pressure from conscious mice and corresponding cardiac hypertrophy (inset). (D) Mean heart rate from conscious mice before and during Iso treatment, and percent Iso-induced change of heart rate (normalized to mean of days –3 to 0). (E and F) No effect of heart rate reduction on cardiac hypertrophy indexes by treatment with Ivabradine before and during (E) or only during (F) Iso treatment.

Table 2 Cardiac function analysed in working heart apparatus

Parameters	WT (n=9)	TRPC1/C4-DKO (n=10)	P-value
Heart rate (BPM)	418 ± 48.0	425 ± 30.1	0.689
Systolic parameters			
Cardiac output (mL/min)	13.1 ± 2.5	12.7 ± 2.6	0.718
Ejection fraction (%)	54.2 ± 9.8	56.5 ± 9.6	0.599
dP/dt _{max} (mmHg/s)	5298 ± 732	5240 ± 574	0.850
End-systolic volume at 100 mmHg (μL)	42.1 ± 10.6 (n=8)	34.4 ± 7.5 (n=9)	0.102
ESPVR			
E _{es} (mmHg/μL)	2.2 ± 0.8 (n=8)	3.1 ± 1.0 (n=9)	0.079
V _o (μL)	9.6 ± 34.3	–6.0 ± 43.0	0.427
Diastolic parameters			
Time constant of relaxation (τ) during isovolumetric diastole (ms)	9.8 ± 0.7	11.0 ± 0.8	0.005
End-diastolic volume (μL)	59.8 ± 14.5	53.6 ± 10.2	0.293
dP/dt _{min} (mmHg/s)	–4232 ± 524	–3963 ± 502	0.269
EDPVR			
k (mmHg)	0.179 ± 0.438	0.051 ± 0.065 (n=9)	0.398
β (μL ^{–1})	0.086 ± 0.039	0.108 ± 0.032	0.211

ESPVR, end-systolic pressure–volume relationship; E_{es}, end-systolic elastance; V_o, volume axis intercept; EDPVR, end-diastolic pressure–volume relationship; β, stiffness constant.

unaltered, indicating that global as well as local Ca^{2+} transport systems were unchanged.

In myocytes from TRPC1/C4-DKO mice systolic Ca^{2+} peaks were reduced after acute stimulation with Iso or AngII. However, during Iso stimulation, the relative amplitude increase was unaltered, indicating that reduced peak Ca^{2+} solely resulted from the reduced diastolic levels. In contrast, AngII-evoked relative increases of the amplitude were reduced in TRPC1/C4-DKO mice suggesting that the TRPC1/C4-dependent Ca^{2+} entry pathway was activated by AngII, most likely via Gi/G_q -PLC signalling. Thus, the reduced hypertrophy for both *in vivo* stimulation regimes can be explained by a reduction in the absolute levels of diastolic and systolic Ca^{2+} in TRPC1/C4-DKO cardiomyocytes. TRPC3/C6 appear not to be part of BGCE; accordingly, diastolic and systolic Ca^{2+} levels were unaltered under basal conditions and during neurohumoral stimulation in cardiomyocytes from TRPC3/C6-DKO. Wu *et al.* used a common protocol to activate store-operated Ca^{2+} entry in non-excitable cells following SERCA inhibition.⁹ They found that expression of negative dominant variants of TRPCs suppressed this Ca^{2+} entry in ventricular cardiomyocytes, but Ca^{2+} homeostasis was not analysed in beating cardiomyocytes. Our results emphasize the role of TRPC1/TRPC4 but not of TRPC3/TRPC6 for the BGCE pathway described here contributing to Iso- and AngII-induced elevations of diastolic and systolic Ca^{2+} concentrations in cardiomyocytes and cardiac hypertrophy, and suggest an explanation why in TRPC3/C6-DKO mice development of cardiac hypertrophy is not reduced.

Transgenic models over-expressing TRPC proteins under a cardiomyocyte promoter implicated that TRPCs other than TRPC1/TRPC4 mediate hypertrophy development.^{9,11,12} Nevertheless, over-expression of TRPC variants may well execute their effects by interaction and functional interference with endogenous TRPC channel proteins in an off-target manner given the considerable amino acid sequence identity of the various TRPC proteins. In our models, the lack of TRPC3/C6 can apparently be compensated by other TRPC proteins and/or TRPC-independent pathways but the relevance of TRPC3/TRPC6 may be more prominent in a model on the C57Bl/6J genetic background⁸ in which nicotinamide nucleotide transhydrogenase inactivation²⁰ may lead to accumulation of reactive oxygen species that enhance the activity of TRPC6-containing channels.²¹ Nevertheless, our findings do not preclude a role of TRPC3/C6 for other cardiac remodelling processes, e.g. during atrial fibrillation,²² Klotho²³ absence or in scar formation after myocardial infarction.^{24,25} Moreover, we cannot exclude that acute pharmacological inhibition may be efficient for reducing cardiac hypertrophy as reported for the compound Pyr3²⁶; however, Pyr3 also blocks ORAI1-mediated store-operated Ca^{2+} entry²⁷ which mediates growth of cultured cardiomyocytes.²⁸ Our experiments demonstrate that development of cardiac hypertrophy is not reduced in TRPC1- or TRPC4-single KO mice. Seth *et al.*¹⁰ showed a reduced hypertrophic response induced by pressure overload or AngII administration in TRPC1-KO mice; however, their induction protocols and genetic background of the TRPC1-KO mice were different from ours.

We further addressed the question whether TRPCs in non-cardiac tissues play a role in the development of cardiac hypertrophy. Both, TRPC1 and TRPC4 are expressed in the kidney, suggesting that they may regulate blood pressure and/or renin

secretion; however, we ruled out both possibilities. Additionally, the reduced Iso-evoked chronotropic response following inactivation of TRPC1/TRPC4, which may be caused by the lack of their modulatory role on sinoatrial node pacemaking²⁹ is very unlikely to affect hypertrophy development since reduction of positive chronotropy using Ivabradine did not protect from development of cardiac hypertrophy as suggested by *in vitro* models.¹⁹ Additionally, in TRPC1/C4-DKO basal cardiac contractility assessed by echocardiography and working heart showed no compromising differences. In contrast, we demonstrate that TRPC1/TRPC4 inactivation protected against cardiac dysfunction evoked by chronically increased afterload.

Analysis of downstream signalling pathways revealed that the up-regulation of RCAN1-4 and myomaxin expression in the heart is mitigated in the absence of TRPC1/C4 implicating that TRPC1/C4-mediated BGCE contributed to the activation of the calcineurin-NFAT pathway. Myomaxin is a transcriptional target of MEF2a which acts as an integrator of multiple Ca^{2+} -dependent signalling molecules including calcineurin and CaMKII³⁰ in the regulation of transcriptional activity.³¹ Although the exact signalling cascade activated by BGCE needs to be explored, our results indicate that BGCE contributes to transcriptional reprogramming of the stressed myocardium including up-regulation of hypertrophy-associated foetal and collagen genes and development of interstitial fibrosis emphasizing its relevance for maladaptive cardiac remodelling processes.

In conclusion, TRPC1 and TRPC4 together form a novel BGCE which determines diastolic and systolic Ca^{2+} as well as their modulation by neurohumoral stimulation in beating cardiomyocytes. It is essential for the development of cardiac hypertrophy and fibrosis in mice. Because the deletion of either TRPC1 or TRPC4 alone appears to be compensated by TRPC4 or TRPC1, respectively, genetic strategies or small molecules specifically targeting the TRPC1/C4-containing Ca^{2+} entry channel complexes in the heart might represent a promising approach to ameliorate the development of pathological cardiac remodelling *in vivo* without altering blood pressure or cardiac contractility.

Supplementary Material

Supplementary Material is available at *European Heart Journal* online.

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Authors' contributions

J.E.C.L., L.K., M.F., and P.L. conceived and designed the research; J.E.C.L., Q.T., K.H., L.S., J.C.L., J.C.R., T.H., M.O., S.M., I.M., S.E.P., W.T., L.K., F.S., and P.L. acquired the data; A.D. and L.B. contributed material; J.E.C.L., J.C.R., I.M., F.S., M.F., and P.L. performed statistical analysis; M.F., V.F., L.K., and P.L. handled funding and supervision; J.E.C.L., M.F., and P.L. drafted the manuscript; and J.E.C.L., J.C.L., U.L., L.B., V.F., M.F., and P.L. made critical revision of the manuscript for key intellectual content.

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