Regulation of murine cardiac contractility by activation of $\alpha_{1A}$-adrenergic receptor-operated $\text{Ca}^{2+}$ entry

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Aims

Sympathetic regulation of cardiac contractility is mediated in part by $\alpha_1$-adrenergic receptors (ARs), and the $\alpha_{1A}$-subtype has been implicated in the pathogenesis of cardiac hypertrophy. However, little is known about $\alpha_{1A}$-AR signalling pathways in ventricular myocardium. The aim of this study was to determine the signalling pathway that mediates $\alpha_{1A}$-AR-coupled cardiac contractility.

Methods and results

Using a transgenic model of enhanced cardiac $\alpha_{1A}$-AR expression and signalling (1A-H mice), we identified a receptor-coupled signalling pathway that enhances $\text{Ca}^{2+}$ entry and increases contractility. This pathway involves $\alpha_{1A}$-AR-activated translocation of Snapin and the transient receptor potential canonical 6 (TRPC6) channel to the plasma membrane. In ventricular cardiomyocytes from 1A-H and their non-transgenic littermates (or WTs), stimulation with $\alpha_{1A}$-AR-specific agonists resulted in increased $[\text{Ca}^{2+}]_i$, which was dose-related and proportional to the level of $\alpha_{1A}$-AR expression. Blockade of TRPC6 inhibited the $\alpha_{1A}$-AR-mediated increase in $[\text{Ca}^{2+}]_i$ and contractility. External $\text{Ca}^{2+}$ entry, underlying the $[\text{Ca}^{2+}]_i$ increase, was not due to store-operated $\text{Ca}^{2+}$ entry but to a receptor-operated mechanism of $\text{Ca}^{2+}$ entry resulting from $\alpha_{1A}$-AR activation.

Conclusion

These findings indicate that $\text{Ca}^{2+}$ entry via the $\alpha_{1A}$-AR-Snapin-TRPC6-pathway plays an important role in physiological regulation of cardiac contractility and may be an important target for augmenting cardiac performance.

Keywords

$\alpha_{1A}$-AR • TRPC • Cardiac contractile function • Cell signalling • Calcium cycling

1. Introduction

Adrenergic receptor (AR) activation in response to sympathetic nervous system stimulation has a major role in regulating cardiac function.$^{1,2}$ Cardiomyocytes (CMs) express both $\alpha$- and $\beta_{1,2}$-ARs. In normal adult hearts, cardiac inotropy and chronotropy are predominantly regulated by $\beta_1$-ARs.$^1$ Under pathological conditions, however, e.g. myocardial ischaemia or hypertrophy, $\alpha_1$-ARs function in a compensatory fashion to maintain cardiac inotropy when $\beta_1$-ARs are down-regulated.$^{1,3}$ and uncoupled from G proteins and effectors.$^1$ In addition, although implicated in the pathogenesis of hypertrophy,$^5$ $\alpha_1$-ARs, particularly the $\alpha_{1A}$-subtype, are important in both developmental CM growth and survival.$^{5,6}$

Cardiac $\alpha_1$-AR stimulation enhances contractility and causes small but significant increases in $[\text{Ca}^{2+}]_i$, but underlying mechanisms remain incompletely understood. It has been suggested that $\alpha_1$-AR-activation is associated with coupling to the $G_{q11}$ family of heterotrimeric G proteins and phospholipase C $\beta$ (PLC$\beta$) isoform-activation,$^7$ resulting in phosphatidylinositol(4,5)-bisphosphate hydrolysis to inositol(1,4,5)-trisphosphate ($IP_3$) and diacylglycerol (DAG). This results in $\text{Ca}^{2+}$ mobilization from intracellular stores and $\text{Ca}^{2+}$ membrane influx.$^8$ Elevated $[\text{Ca}^{2+}]_i$, might also be due to (i) store-operated
channels (SOCs) activated, for example, by IP3-induced Ca2+ depletion of internal stores or (ii) via receptor-operated channels (ROCqs). It is unclear whether these pathways are major contributors to CM α1A-AR signalling, since PLCβ activity and IP3 generation as well as IP3-receptor (IP3R) expression are generally low in CMs. Some studies suggest that TRPCs act as SOCs; others have shown that they can also function as ROCqs. In PC12 cells, interactions between TRPC6 and the adaptor protein, Snapin, regulate α1A-AR-mediated Ca2+ influx—Snapin being a SNARE-associated modulatory protein involved in exocytosis and cytokinesis.

Here, we utilized a transgenic mouse model with cardiac-restricted α1A-AR overexpression to show that TRPC6–Snapin interactions determine α1A-AR signalling, both in isolated CMs and in vivo. In this transgenic model, mice with four-fold α1A-AR overexpression (α1A-L) are phenotypically normal, while mice with 66-fold (α1A-M) and 170-fold (α1A-H) overexpression display expression-related increases in systolic contractile function. Interestingly, despite increased α1A-AR expression, none of these mice showed cardiac hypertrophy under baseline conditions, and hypertrophic responses to pressure overload were similar to those in wild-type (WT) littermates. Moreover, while enhanced α1A-AR-drive in the α1A-M line improved survival in response to myocardial infarction or pressure overload, the α1A-H line is susceptible to sudden cardiac death characterized by a rapid reduction in QRS amplitude in the absence of significant brady- or tachyarrhythmias. Here, we show that stimulation of α1A-ARs results in a dynamic interaction of Snapin and TRPC6 resulting in receptor-operated Ca2+ entry (ROCE), and that blockade of this pathway prevents sudden cardiac death in α1A-H mice. Although an increase in [Ca2+]i upon α1A-AR activation is barely detectable in normal CMs, this α1A-AR-coupled Ca2+ entry pathway is associated with augmented contractility even in WT hearts. Furthermore, enhancement of this signalling pathway underlies both the hypercontractility of α1A-H mice and the sudden death observed in this transgenic model.

2. Methods

2.1 Animals

Transgenic male mice with cardiac-specific overexpression (α1A-H, 170-fold; α1A-M, 66-fold) of the rat α1A-AR on a FVB/N genetic background, and their non-transgenic littermates (or WTs), were studied at 2–4 months of age. All experimental procedures were approved by the Garvan/St Vincent’s Hospital Animal Ethics Committee, in accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The investigation conforms with the guidelines of the Australian Code of Practice for the Care and Use of Animals (NIH Publication no 85–23, revised 1996).

2.2 Development of pore inhibitory anti-TRPC1 and TRPC6 antibodies

Rabbit anti-TRPC1 and anti-TRPC6 antibodies, raised (Peptide Specialty Laboratories GmbH, Heidelberg, Germany) against the putative pore-forming loop of these TRPCs, were immunopurified using the respective peptide immunogens coupled to CNBr-activated agarose (see Supplementary material online, Methods for details).

2.3 Production of shRNA-encoding adenoviruses

Oligonucleotide sequences for shRNA1-Snapin, shRNA2-Snapin, or shRNA scrambled (see Supplementary material online, Methods) were cloned into the multiple cloning site of pAdTrack-si, and recombinant adenoviruses were generated as described.

2.4 Reverse transcriptase–polymerase chain reaction

Snapin, TRPC1, TRPC6, and porphobilinogen deaminase cDNAs were generated from mouse total RNA as detailed in Supplementary material online, Methods.

2.5 Immunoblot analysis

Western blotting was performed using antibodies to Snapin, TRPC1, or TRPC6 (see Supplementary material online, Methods).

2.6 Cell lines, culture, and transfection

HEK293 and COS cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (see Supplementary material online, Methods) and transfected using Lipofectamine 2000 (Invitrogen).

2.7 Isolation, cell culture, and adenoviral infection of ventricular adult mouse CMs

α1A-H and α1A-M and WT mice were anesthetized with Nembutal (60 mg/kg, ip; anaesthesia depth was monitored by limb withdrawal using toe pinching), a thoracotomy was performed and left ventricular CMs isolated from the hearts by enzymatic retrograde perfusion, as detailed in Supplementary material online, Methods. CMs were incubated for 1 h at 37°C and 2% CO2 in plate media (MEM media containing 10% FCS, 10 mM BDM, 2 mM Penicillin/Streptomycin, 2 mM Glutamine). CMs were then washed two times with culturing media (MEM media containing 0.5% FCS, 2 mM penicillin/streptomycin, 2 mM glutamine) and cultured in culturing media at 37°C and 2% CO2, with or without adenovirus at MOI 100, for 96 h.

2.8 Immunocytochemistry

CMs cultured on laminin-coated glass coverslips were stimulated with methoxamine (100 μmol/L), A61603 (100 mmol/L) and/or prazosin (100 nmol/L), fixed, permeabilized and stained for Snapin, TRPC1, or TRPC6, as detailed in Supplementary material online, Methods.

2.9 Transmission electron microscopy and immunogold labelling

Cardiac tissue sections were stained with TRPC1 or TRPC6 with 10 nm colloidal-gold-conjugated antibody, and for Snapin or caveolin antibodies with 20 nm colloidal-gold-conjugated antibody, as detailed in Supplementary material online, Methods. Pictures were taken at ×50 000 magnification.

2.10 Measurement of [Ca2+]i

Ca2+ recordings were performed either using batches of isolated, fura-2 AM-loaded CMs in a cuvette to quantify mean fluorescence, or using single-isolated fluo-4 AM-loaded CMs monitored by confocal Ca2+ microscopy (for details, see Supplementary material online, Methods).

2.11 Isolated heart perfusions

Animals were anaeasthetized (Nembutal, 60 mg/kg, ip) and a thoracotomy was performed, and hearts from α1A-H or WT mice were isolated for Langendorff perfusion (detailed in Supplementary material online, Methods). Seven to eight animals were analysed per group.
2.12 Co-immunoprecipitation

Animals were anesthetized (1.5% isofluorane in medical grade O2 delivered by nose cone; anaesthesia depth was monitored by limb withdrawal using toe pinching) and a 1.4 Fr Millar catheter introduced in the left carotid artery to monitor arterial blood pressure. After measurement of baseline parameters, saline or PE (WT 100 g/kg; α1A-AR-H 3.3 μg/kg in 25 μL) was delivered over 1 min into the left jugular vein with a 100 μL Hamilton syringe using an sp200i syringe pump. Prazosin (0.5 mg/kg, in 25 μL, ip, 1 min incubation) was administered as a bolus in a volume of 1 mL/kg as indicated. Hearts were quickly extracted at the times indicated, membrane fractions were prepared, solubilized, pre-cleared, incubated with Snapin-FL-antibody covalently coupled to G-sepharose, and proteins eluted for SDS–PAGE electrophoresis as described in Supplementary material online, Methods.

2.13 Data analysis

Results shown are means ± 1SD. Statistical differences were determined by two-way analysis of variance (two-way ANOVA) for quantification of intensity staining and Ca2+ recordings and one-way ANOVA for isolated hearts followed by a pairwise comparison, with P ≤ 0.05 being considered significant.

3. Results

3.1 Endogenous expression and subcellular localization of TRPC6, TRPC1, and Snapin in CMs and heart tissue

Immunoblot (Figure 1A, left panel) and densitometric analyses (data not shown) revealed Snapin, TRPC6, and TRPC1 expression in isolated CMs and in myocardium of α1A-H and WT mice. Snapin and TRPC6 expression levels were more marked in the myocardium, suggesting that their expression is not limited to CMs, but also occurs in non-CMs. In contrast, expression of TRPC1, a well-characterized SOC, was similar in myocardium and in CMs, indicating predominant CM expression. Transmission electron microscopy (TEM) of immunogold-labelled α1A-H and WT myocardium showed co-localization of Snapin, TRPC6, and TRPC1 in the plasma membrane and the t-tubules; the latter evident from caveolin-3 immunolabelling (Figure 1B). Specificity of antibody staining was confirmed by blockade of immunoblotting (Figure 1A, right panel) with the peptide immunogens used to generate anti-TRPC6 and anti-TRPC1 antibodies.

3.2 Dynamic interaction of α1A-AR, Snapin, and TRPC6 in vitro and in vivo

Prior to CM stimulation with a commonly used α1A-AR agonist, methoxamine, TRPC6, and Snapin were evident in both the plasma membrane and t-tubules as well as in the cytosol of WT CMs (Figure 2A–C), with slightly higher plasma membrane vs. intracellular localization in unstimulated α1A-H CMs (Figure 2B and C). α1A-AR activation resulted in translocation of Snapin and TRPC6 from the t-tubules to the plasma membrane in both WT and α1A-H CMs, as evident by the loss of tubular staining and a weaker striation pattern (Figure 2A). This translocation was abrogated by pre-treatment with the α1A-AR-specific antagonist, prazosin. In WT CMs, recruitment to the plasma membrane peaked at 5–10 min after α1A-AR activation (Figure 2B), whereas in α1A-H CMs, plasma membrane recruitment was faster (peaking at 2 min) (Figure 2C). In contrast to TRPC6, no significant plasma membrane translocation of TRPC1 was observed with α1A-AR activation of WT or α1A-H CMs (see Supplementary material online, Figure S1).

Next, we investigated whether the interaction of α1A-AR, Snapin, and TRPC6 observed in vitro was also operative in vivo. Hearts were rapidly excised from α1A-H and WT mice after administration of phenylephrine (PE) to activate α1A-ARs or saline. Immuno precipitation of solubilized membranes with an anti-Snapin antibody showed that α1A-AR and TRPC6 (but not TRPC1; data not shown) co-immunoprecipitated with Snapin in both α1A-H mice and WT hearts, even basally after saline administration, although this interaction was greater in α1A-H mice (see Supplementary material online, Figure S2). In the WT, co-immunoprecipitation was maximal at 1.5 min and waned by 7 min after PE administration (see Supplementary material online, Figure S2). In both genotypes, this interaction was increased after α1A-AR activation, although to a greater extent in α1A-H mice (see Supplementary material online, Figure S2B). These data indicate a dynamic interaction between α1A-AR, Snapin, and TRPC6 in vivo.

3.3 Increased [Ca2+]i in α1A-M and α1A-H relative to WT CMs after α1A-AR activation

To provide more robust evidence for an interaction between α1A-AR, Snapin, and TRPC6, we used the highly selective α1A-AR agonist, A61603, in subsequent studies. Consistent with very low α1A-AR expression in WT CMs, receptor stimulation with A61603 resulted in only a slight increase in [Ca2+]i, in these cells, which was only significant at the highest A61603 dose (30 nM; Figure 3A). In contrast, dose-dependent tonic increases in [Ca2+]i, directly related to the level of α1A-AR overexpression, were evident in α1A-M and α1A-H CMs, and were already highly significant with 1 nM A61603. At the highest (30 nM) A61603 dose, increases in [Ca2+]i, of up to ~1–1.5 μM were observed (Figure 3A). Similar increases in [Ca2+]i, have also been observed with field stimulation of CMs. To confirm the specificity of the A61603-mediated increase in [Ca2+]i, we demonstrated that it could be abolished by pre-treatment with the α1A-AR-selective antagonist, KMD3213 (Figure 3B).

3.4 Increase in [Ca2+]i, is triggered by ROCE upon α1A-AR activation and requires TRPC6 and Snapin

To investigate whether α1A-AR-stimulated tonic increases in [Ca2+]i, are due to Ca2+ influx through SOCs or ROCs, we evaluated [Ca2+]i, both in batches of isolated CMs to carefully quantify the [Ca2+]i, signal, as well as in single-isolated cells. As shown in Figure 3C, addition of non-specific TRPC channel- and ROC/SOC inhibitors, La3+ or gentamicin, to batches of CMs from α1A-H or WT mice suppressed α1A-AR-mediated increases in [Ca2+]i, whereas inhibition of store-operated Ca2+ entry (SOCE) with 2-APB, or IP3-mediated signalling with xestospongin C, had no effect. To demonstrate ROCE, CMs were treated with ROC/SOC inhibitors, La3+, and gentamicin, as well as the SOC inhibitor, 2-APB, after initial depletion of the sarcoplasmic reticulum (SR) Ca2+. ATPase using cyclopiazonic acid (CPA) and/or after caffeine-induced release from the ryanodine-sensitive Ca2+ pool. As shown in Figure 3D–G, increases in [Ca2+]i, resulting from enhanced Ca2+ influx due to α1A-AR stimulation with A61603 were observed after CPA pre-treatment, which depletes intracellular stores and
pre-activates SOCs. Of interest, similar responses were observed in studies of single-isolated CMs where cells were pre-treated with both CPA and caffeine (see Supplementary material online, Figure S3A). Moreover, in both studies of CM batches (Figure 3D and E) and single-isolated CMs (see Supplementary material online, Figure S3A), α1A-AR-stimulated Ca^{2+} influx was observed only if Ca^{2+} was present in the extracellular medium. Influx was not evident in the absence of extracellular Ca^{2+} but could be restored by the addition of external Ca^{2+} to α1A-H CMs (Figure 3F and G; see Supplementary material online, Figure S3B). This was most evident in single-isolated α1A-H CMs where SOCE activation after store depletion only caused a minor [Ca^{2+}] increase when compared with the large increase observed after A61603 administration. The latter was more transient if cells were bathed in 1.8 mM Ca^{2+} throughout, but was marked and sustained when A61603 was applied under Ca^{2+}-free conditions and then 1.8 mM Ca^{2+} reintroduced to the external medium (see Supplementary material online, Figure S3A and B). Note that the sharp Ca^{2+} peaks in these recordings correspond to the short global transients following caffeine application or represent spontaneous Ca^{2+} waves.

To further delineate whether the A61603-mediated increase in Ca^{2+} was due to SOCE or ROCE, α1A-ARs on single-isolated CMs were blocked with the α1A-selective antagonist, KMD3213, after depletion of SR Ca^{2+} stores, but before the addition (~150 s later) of Ca^{2+} to the external medium (see Supplementary material online, Figure S3C). In this situation, no increase in cytoplasmic Ca^{2+} was observed (see Supplementary material online, Figure S3C), arguing against a mechanism involving activation of SOCE, which would be expected to result in an influx of Ca^{2+}, independent of α1A-AR activity. This scenario largely excludes SOCE, which is

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**Figure 1** Snapin, TRPC6, and TRPC1 expression in isolated CMs and heart tissue and localization in heart sections from α1A-H and WT mice. (A) Solubilized membranes (120 µg) from WT or α1A-H CMs or heart tissue were fractionated on 6% SDS–PAGE for TRPC6 (~107 kDa) and TRPC1 (~93 kDa) or 15% SDS–PAGE for Snapin (~15 kDa) followed by immunoblotting with anti-Snapin, or anti-TRPC6, or anti-TRPC1 antibody in the absence (−) (left panels) or presence (+) (right panels) of their specific peptide immunogen. As loading controls, solubilized membranes were similarly evaluated with anti-GAPDH antibody. (B) Double immunogold labelling with anti-TRPC6 (small—10 nm gold particles, arrowhead) and anti-Snapin (large—20 nm gold particles, arrow) antibodies. I and II show co-localization of TRPC6 and Snapin along the plasma membrane (I), and at the ends of sarcomere Z-lines localized to t-tubules. T-tubule localization of TRPC6 was confirmed by double immunogold labelling of anti-TRPC6 (10 nm particles) and t-tubule-specific anti-caveolin-3 antibodies (20 nm particles) (III and IV). Both proteins co-localize at the t-tubules (arrows). TRPC1 showed a similar pattern of expression to TRPC6 (not shown). BV, blood vessel; Nu, nucleus. Scale bars: I, II, and IV = 200 nm; III = 500 nm. Images were obtained using a JEOL1400 TEM microscope at ×50 000.
known to be restricted to an α1A-AR-activation-independent Stim1–Orai1 interaction. To verify that α1A-AR-mediated Ca\(^{2+}\) influx is dependent on TRPC6 activity, cells were pre-incubated with anti-TRPC6 antibody and then α1A-ARs were activated after store depletion in Ca\(^{2+}\)-free medium (see Supplementary material online, Figure S3D). This resulted in a continued decline in fluo-4 signal, most likely due to further Ca\(^{2+}\) efflux as a result of continued activity of sodium–calcium exchangers and plasma membrane Ca\(^{2+}\)-ATPases. After stimulation of α1A-AR with A61603 and restoration of external Ca\(^{2+}\), cytoplasmic Ca\(^{2+}\) failed to increase. Taken together, these results indicate that in α1A-H CMs the increased [Ca\(^{2+}\)] observed with activation of the α1A-AR is mediated by ROCE, with no or only minor involvement of SOCE.

Furthermore, this ROCE requires full functional availability of TRPC6. Blockade of TRPC6 with the anti-TRPC6 antibody dose-dependently inhibited [Ca\(^{2+}\)] increase resulting from α1A-AR activation in cells studied in batches (Figure 4A) and in single-isolated CMs using one effective antibody concentration (see Supplementary material online, Figure S3D). In contrast to the very pronounced effect of the anti-TRPC6 antibody, the anti-TRPC1 antibody had a much smaller effect on A61603-mediated increases in cytoplasmic Ca\(^{2+}\) in α1A-H CMs (Figure 4A). Moreover, neither TRPC1 nor TRPC6 pre-immune serum affected α1A-AR-mediated increases in [Ca\(^{2+}\)] (see Supplementary material online, Figure S4). Snapin knockdown also attenuated α1A-AR-mediated Ca\(^{2+}\) entry. This is shown in Figure 4B, where Snapin knockdown was achieved by infection of CMs with a lentivirus expressing a shRNA against Snapin.
with either of two adenoviruses encoding anti-Snapin shRNA. In contrast, α1A-AR-mediated Ca\textsuperscript{2+} entry was evident in uninfected CMs or in cells infected with an adenovirus encoding a scrambled anti-Snapin shRNA. Functionality and specificity of anti-Snapin-specific shRNA-adenoviruses was demonstrated by reverse transcriptase–polymerase chain reaction and immunoblotting (see Supplementary material online, Figure S5), where the steady-state level of Snapin mRNA was markedly suppressed 96 h after adenoviral infection with either Snapin-specific shRNA adenovirus, as was Snapin protein expression. In contrast, in CMs infected with adenovirus encoding scrambled anti-Snapin shRNA, Snapin transcript, and protein expression were unaltered. Snapin residues targeted by the two anti-Snapin shRNAs (shRNA1-Snapin and shRNA2-Snapin) are indicated in Supplementary material online, Figure S5E. Taken together, these findings indicate that both Snapin and TRPC6 are required for α1A-AR-stimulated Ca\textsuperscript{2+} entry.

3.5 Snapin specifically couples TRPC6 to the α1A-AR

G\textsubscript{α11}-coupled angiotensin-II type 1 receptors (AT\textsubscript{1}) are expressed in the heart and can activate TRPC6 in CMs.\textsuperscript{30} As shown in Figure 4C, Angiotensin II (Ang II, 100 nM) stimulation of α1A-H and WT CMs-induced equivalent increases in [Ca\textsuperscript{2+}], that were markedly reduced by TRPC6 inhibition, but not by Snapin knockdown. This indicates that although α1A-AR-mediated TRPC6 activation is Snapin-dependent, TRPC6 activation by another G\textsubscript{q}-coupled G protein-coupled receptor (GPCR), that for Ang II, is not.

3.6 Essential role of PLCβ in α1A-AR-stimulated Ca\textsuperscript{2+} entry

Inhibition of PLCβ by U-73122 (but not its inactive enantiomer, U73343) suppressed α1A-AR-stimulated increases in [Ca\textsuperscript{2+}], in both
α1A-H and WT CMs (Figure 4D). Also, increasing DAG levels by the addition of the membrane permeable DAG analogue, 1-oleoyl-2-acetyl-sn-glycerol (OAG), or by the inhibition of DAG metabolism with R59022, a DAG lipase inhibitor, increased \([\text{Ca}^{2+}]_i\) and enhanced the increase in \([\text{Ca}^{2+}]_i\) observed with a1A-AR activation alone in WT CMs (Figure 4E), which is likely due to direct activation of TRPC6 by DAG.31 However, despite administration of OAG at the same concentration that alone increased \([\text{Ca}^{2+}]_i\), in α1A-H and WT CMs, Snapin knockdown, or TRPC6 blockade suppressed A61603-stimulated increases in \([\text{Ca}^{2+}]_i\), in both α1A-H and WT CMs (Figure 4E). These findings indicate that α1A-AR/Snapin/TRPC6-mediated \([\text{Ca}^{2+}]_i\) entry is augmented by PLCβ-mediated DAG production via an effect at the level of TRPC6.

3.7 Increases in cardiac contractility in WT and α1A-AR-H mice and sudden cardiac death of α1A-H mice is prevented by blockade of α1A-ARs or TRPC6

We reported previously that α1A-H mice die prematurely of sudden cardiac death manifested by a progressive reduction in QRS amplitude and then in left ventricular pressure.20 To investigate whether this
response involved Ca$^{2+}$ entry via $\alpha_{1A}$-AR-stimulated TRPC6 activation, we used isolated perfused hearts from $\alpha_{1A}$-H mice. As shown in Figure 5B, activation of $\alpha_{1A}$-ARs with A61603 mimicked the sudden cardiac death response observed in intact animals, and A61603 washout restored heart function. Administration of either KMD3213 (data not shown) or anti-TRPC6 antibody, but not anti-TRPC1 antibody (data not shown), before A61603-stimulation, prevented the hypercontractile response in both $\alpha_{1A}$-H and WT hearts, and completely protected $\alpha_{1A}$-H hearts from sudden cardiac death (Figure 5A and C). The cardiac haemodynamics in WT and $\alpha_{1A}$-H Langendorff-perfused hearts are given in Supplementary material online, Table S1. These findings provide evidence that $\alpha_{1A}$-AR-mediated activation of TRPC6 is a physiologically relevant pathway underlying $\alpha_{1A}$-AR-mediated contractile responses in WT mice and is pathophysiologically involved in sudden cardiac death of $\alpha_{1A}$-H mice.

4. Discussion

Myocardial $\alpha_{1A}$-AR signalling pathways remain poorly defined, partly because of low levels of $\alpha_{1A}$-AR expression and, thus, limited signal output in normal heart under physiological conditions. The signalling pathway mediating positive inotropic effects of $\alpha_{1A}$-ARs has been studied for 30 years but remains enigmatic. The increase in [Ca$^{2+}$], associated with $\alpha_{1A}$-AR activation in the normal heart is relatively small compared with the increase in force, suggesting that a receptor-stimulated increase in Ca$^{2+}$ sensitivity is importantly involved in receptor-coupled increase in contractility. However, in skinned fibre preparations, pCa$^{2+}$-force curves are unaltered between $\alpha_{1A}$-H and WT preparations (Z.-Y. Yu, J.-C. Tan, A.C. McMahon, S.E. Imsa, X.-H. Xiao, S.H. Kesteven, M.E. Reichelt, M.C. Mohl, D. Fatkin, D.G. Allen, S.I. Head, R.M. Graham, M.P. Fenelley, unpublished observations). Here, we took advantage of an $\alpha_{1A}$-H transgenic mouse model with enhanced cardiac $\alpha_{1A}$-AR expression and activity to investigate $\alpha_{1A}$-AR-coupled signalling. Based on our findings, we present a model for $\alpha_{1A}$-AR-coupled cardiac Ca$^{2+}$ entry involving redirection and activation of TRPC6 from the cytoplasm to the plasma membrane via interaction with Snapin. This in turn enhances cardiac contractility. TRPC6-mediated Ca$^{2+}$ entry is also partially modulated by $\alpha_{1A}$-AR-stimulated activation of G$_{q/11}$, and, thus, PLC$b$, which results in DAG production that independently activates TRPC6 in the plasma membrane (Figure 6). In support of this novel $\alpha_{1A}$-AR-coupled signalling pathway, we show: (i) interaction of $\alpha_{1A}$-AR-Snapin-TRPC6, which is increased in vivo in the absence of $\alpha_{1A}$-agonist in $\alpha_{1A}$-H relative to WT hearts, and is further enhanced by $\alpha_{1A}$-AR activation; (ii) $\alpha_{1A}$-AR-mediated translocation of TRPC6 and Snapin from the cytosol to the plasma membrane; (iii) dose-related increases in CM [Ca$^{2+}$], proportional to the level of $\alpha_{1A}$-AR expression and sensitive to $\alpha_{1A}$-AR inhibition. TRPC6 blockade, or Snapin knockdown; (iv) increased $\alpha_{1A}$-AR-stimulated CM [Ca$^{2+}$], sensitive to inhibition of PLC and augmented by inhibition of DAG metabolism or by administration of the DAG analogue OAG and (v) $\alpha_{1A}$-AR-mediated increases in [Ca$^{2+}$], mediated by activation of ROCO rather than SOCE, since (a) inhibition of SOC activity or of IP$_3$Rs and SERCA did not block $\alpha_{1A}$-AR-stimulated increases in [Ca$^{2+}$], and (b) depletion of SR stores in the absence of extracellular Ca$^{2+}$ inhibited $\alpha_{1A}$-AR-mediated Ca$^{2+}$ influx and was restored by the addition of Ca$^{2+}$ only when the $\alpha_{1A}$-AR–TRPC6 interaction was preserved; and (vi) most intriguingly, antibody-mediated blockade of TRPC6 not only inhibited $\alpha_{1A}$-AR-mediated increases in cardiac contractility in WT and $\alpha_{1A}$-H hearts, but also prevented $\alpha_{1A}$-AR-stimulated Ca$^{2+}$-induced sudden cardiac death in $\alpha_{1A}$-H mice. Studies in vascular smooth muscle and other non-cardiac tissues such as brain and neuronal cell lines support our finding that TRPC6 serves as an $\alpha_{1A}$-AR-activated Ca$^{2+}$-permeable cation channel that mediates increased [Ca$^{2+}$] in non-cardiac cells, its role in CMs is questionable, since IP$_3$ production is not increased in $\alpha_{1A}$-H mice. Nonetheless, despite the low levels of IP$_3$R expression in CMs, IP$_3$R-mediated increases in nuclear [Ca$^{2+}$] appear to underlie endothelin-1 (ET-1)-stimulated hypertrophy.

Like $\alpha_{1A}$-ARs, AT$_1$-receptors also couple to G$_{q/11}$ and have been reported to stimulate Ca$^{2+}$ entry via TRPC6 activation. However, AT$_1$-receptor coupling to TRPC6 does not involve Snapin. Instead, Snapin appears to be an $\alpha_{1A}$-AR-specific adaptor protein that does

![Figure 5](image-url) TRPC6-blockade prevents A61603-induced contractility in Langendorff-perfused WT hearts, and A61603-induced sudden cardiac death in isolated $\alpha_{1A}$-H hearts. Representative LV systolic pressure (LVP Sys; mmHg/s) and ± dP/dt (mmHg/s) traces from (A) WT, or (B), (C), $\alpha_{1A}$-H hearts, before and after A61603 (100 nmol/L) ± anti-TRPC6 antibody (Ab; 10 µg/mL), administered at the times indicated (arrows). Data are representative of similar responses in seven to eight hearts.
not interact promiscuously with other Gq/11PCRs since (i) Snapin knockdown failed to inhibit Ang-II-stimulated increases in [Ca2+]i, and (ii) a BLAST search of the α1A-AR sequence that specifically interacts with Snapin12 shows no homology with any other GPCR.

Our data show that in CMs, TRPC6 and Snapin co-localize along the plasma membrane and at the end of sarcomere Z-lines, even in the absence of α1A-AR stimulation, suggesting that they interact in a pre-formed complex to facilitate rapid ROCE. Consistent with preferential sarcolemmal localization of TRPC6 observed in CMs from cardiac-specific TRPC6 overexpressing mice,35 we found translocation of Snapin and TRPC6 to the plasma membrane following α1A-AR stimulation. We do not yet know whether α1A-AR stimulation also results in Snapin and TRPC6 translocation to the t-tubules—structures that house important signalling molecules, such as L-type Ca2+ channels that are involved in βAR-mediated excitation–contraction coupling.

Recent findings indicate a role of TRPC channels and TRPC6 in hypertrophy12 but not contraction. Differential localization may have important functional consequences that may underlie the enhanced cardiac contractility mediated by α1A-AR-stimulated Ca2+ entry via TRPC6, and the lack of hypertrophy in α1A-H mice. By analogy, excitation–contraction-induced increases in [Ca2+]i in CMs are restricted to the cytosol, whereas the increase in [Ca2+]i due to ET-1 stimulation is restricted to the nucleus.34 Thus, Ca2+ entry via α1A-AR-stimulated TRPC6 channels may be restricted to juxta-membranous microdomains at sites distinct from those involved in βAR-stimulated Ca2+ entry.26

In addition to physiologically regulating α1A-AR-stimulated cardiac contractility in WT mice, TRPC6, at least to some extent, also appears to be critically involved in the pathophysiology of the sudden cardiac death observed in α1A-H mice observed in response to increased sympathetic activity. Prevention of sudden cardiac death by α1A-AR blockade and independently by TRPC6 blockade indicates that this response is a result of excessive α1A-AR-mediated Ca2+ entry via TRPC6. Excessive Ca2+ is known to inhibit gap junction conductance37—an effect entirely consistent with the progressive reduction in QRS amplitude without arrhythmias in α1A-H mice, but one that contrasts with the ventricular fibrillation and sudden cardiac death observed with store-overload-induced Ca2+ release.38

In vivo, the α1A-H model displays a robust increase in systolic contractility (dP/dtmax) with little change in diastolic relaxation (dP/dtmin) and no hypertrophy.17 Thus, receptor-coupled signalling pathways for contractility and hypertrophy may be spatially and temporally dissociated in CMs, despite the seeming commonality of enhanced [Ca2+]i being required for both. In agreement with this notion is the finding that under physiological conditions, activation of gene transcription programmes can be dissociated from enhanced [Ca2+]i, following activation of the prototypical pro-proliferative receptor, the epidermal growth factor receptor.39

In summary, we have delineated a novel myocardial α1A-AR signalling pathway that provides significant insights into receptor-regulated cardiac contractility and Ca2+ handling in the context of both WT α1A-AR expression, and receptor overexpression. Given that the transgenic model of enhanced α1A-AR activity displays robust hypercontractility but no hypertrophy, our data suggest that modest activation of the α1A-AR-Snapin-TRPC6-pathway may be a promising approach for therapeutically augmenting cardiac performance in clinical settings of impaired cardiac performance, such as myocardial infarction or heart failure.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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