

The TRPM4 channel is functionally important for the beneficial cardiac remodeling induced by endurance training

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Abstract Cardiac hypertrophy (CH) is an adaptive process that exists in two distinct forms and allows the heart to adequately respond to an organism's needs. The first form of CH is physiological, adaptive and reversible. The second is pathological, irreversible and associated with fibrosis and cardiomyocyte death. CH involves multiple molecular mechanisms that are still not completely defined but it is now accepted that physiological CH is associated more with the PI3-K/Akt pathway while the main signaling cascade activated in pathological CH involves the Calcineurin-NFAT pathway. It was recently demonstrated that the TRPM4 channel may act as a negative regulator of pathological CH by regulating calcium entry and thus the Cn-NFAT pathway. In this study, we examined if the TRPM4 channel is involved in the physiological CH process. We evaluated the effects of 4 weeks endurance training on the hearts of *Trpm4*^{+/+} and *Trpm4*^{-/-} mice. We identified an elevated functional expression of the TRPM4 channel in cardiomyocytes after endurance training suggesting a potential role for the channel in physiological CH. We then observed that *Trpm4*^{+/+} mice displayed left ventricular

hypertrophy after endurance training associated with enhanced cardiac function. By contrast, *Trpm4*^{-/-} mice did not develop these adaptations. While *Trpm4*^{-/-} mice did not develop gross cardiac hypertrophy, the cardiomyocyte surface area was larger and associated with an increase of Tunel positive cells. Endurance training in *Trpm4*^{+/+} mice did not increase DNA fragmentation in the heart. Endurance training in *Trpm4*^{+/+} mice was associated with activation of the classical physiological CH Akt pathway while *Trpm4*^{-/-} favored the Calcineurin pathway. Calcium studies demonstrated that TRPM4 channel negatively regulates calcium entry providing support for activation of the Cn-NFAT pathway in *Trpm4*^{-/-} mice. In conclusion, we provide evidence for the functional expression of TRPM4 channel in response to endurance training. This expression may help to maintain the balance between physiological and pathological hypertrophy.

Keywords TRPM4 channel · Cardiac hypertrophy · Endurance training · Calcium entry

Introduction

Cardiac hypertrophy (CH) reflects an adaptive enlargement of the heart in order to maintain or increase its output. CH can be classified by the functional outcomes as either physiological (normal) or pathological (detrimental) (Bernardo et al. 2010; Shimizu and Minamino 2016). Physiological CH develops in response to endurance training or pregnancy. The morphological cardiac growth and associated enhanced cardiac contractility are reversible (Eghbali et al. 2005; Umar et al. 2012; Mann and Rosenzweig 2012). In exercise-induced physiological CH, the canonical intracellular signaling pathway phosphatidylinositol-3

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kinase–protein kinase B (PI3K-Akt) pathway is involved (Kemi et al. 2008; Kim et al. 2008; Ikeda et al. 2009). Upstream stimulation of molecules such as the β -adrenergic receptors, insulin receptor or insulin-like growth factor one activates Akt through PI3K α . Akt, the key molecule for physiological CH, also named protein kinase B, is a serine/threonine kinase, which phosphorylates downstream targets such as glucose transporter (GLUT1) and mTOR (mammalian target of rapamycin). While three isoforms of Akt (Akt1, Akt2 and Akt3) exist, only Akt2 and Akt1 are expressed in heart. It is now well established that Akt1 mediates cardiac growth and that Akt2 is important for cell metabolism (DeBosch et al. 2006). Finally, the major effect of the activation of this signaling pathway in cardiomyocytes is an increase of cell size, inhibition of apoptosis, and regulation of metabolism (Tao et al. 2015).

By contrast, pathological CH develops in response to disease-associated stimuli such as hypertension, myocardial infarction or genetic mutations. Activation of the neuroendocrine system provokes alterations in signal-transduction pathways associated with increased cell size, increased apoptosis, and fibrosis (Bernardo et al. 2010). The literature is profuse on the involvement of the pathway involving the calcium (Ca^{2+}) dependent phosphatase, calcineurin (Cn) and nuclear factor of activated T-cells (NFAT). This signaling pathway is sufficient to induce pathological CH (Molkentin et al. 1998; Wilkins et al. 2004; Wilkins and Molkentin 2004). Cn dephosphorylates NFAT, allowing its nuclear translocation, and thus inducing a hypertrophic gene expression adaption (Wilkins and Molkentin 2004). Ca^{2+} entry in the cardiomyocytes is critically involved in pathological CH (Bers 2008; Cho et al. 2016). It has been hypothesized that members of the transient receptor potential (TRP) channels family could induce signal transduction via Ca^{2+} elevation and Ca^{2+} store refill, prolonging the pathological hypertrophic pathway (Nakayama et al. 2006; Bush et al. 2006; Onohara et al. 2006; Kuwahara et al. 2006; Makarewich et al. 2014). By contrast, physiological CH, induced by exercise or pregnancy, is not associated with an increase of the Cn-NFAT pathway (Wilkins et al. 2004).

The TRPM4 channel (Ca^{2+} -activated TRP subfamily melastatin, member 4) is a voltage-sensitive channel that selectively conducts Na^+ and K^+ but not Ca^{2+} ions thereby negatively regulating Ca^{2+} entry from store-operated Ca^{2+} channels (Launay et al. 2002, 2004; Nilius et al. 2003; Vennekens et al. 2007; Park et al. 2008; Barbet et al. 2008). TRPM4 channel is widely expressed and functional in numerous tissues and has been implicated in Ca^{2+} -dependent functions (Barbet et al. 2008; Guinamard et al. 2010; Earley 2013; Li et al. 2014). This channel is expressed in the left ventricle in various forms of pathological CH (Colquhoun et al. 1981; Guinamard et al. 2002,

2006). In particular, TRPM4 channel expression correlated positively with the heart weight to body weight ratio in spontaneously hypertensive rats while no expression was detected in control rats (Guinamard et al. 2006). Conversely, other groups report no modification of its expression in an experimental murine CH model (Jacobs et al. 2015). A putative protective role of TRPM4 in the development of pathological CH has also been suggested by preventing the activation of the Cn-NFAT pathway (Kecskés et al. 2015).

The role of TRPM4 in physiological CH is currently unknown. In the present study, we investigated the potential role of the TRPM4 channel in the normal adaptive CH in response to endurance training using wild-type mice (*Trpm4*^{+/+}) and *Trpm4* knockout mice (*Trpm4*^{-/-} mice). We found an increased TRPM4 channel expression and function, associated with a functional regulation of Ca^{2+} entry through store-operated channels (SOC), and activation of the PI3K-Akt pathway in *Trpm4*^{+/+} mice in response to endurance training. By contrast, the combination of endurance training and the absence of the TRPM4 channel was associated with increased apoptosis and the activation of the pathological associated CH Cn-NFAT signaling pathway. Our results suggest that expression of TRPM4 channel, due to hypertrophic remodeling, may modulate a balance between physiological and pathological CH signaling pathways.

Results

Functional expression of TRPM4 channel is promoted by endurance training

We investigated whether TRPM4 channel expression could be detected in response to 4 week endurance training (Tr) when compared to sedentary mice (Sed). At the transcript level, assessed by quantitative RT-PCR, TRPM4 channel was detected in the left ventricle (LV) of Sed-*Trpm4*^{+/+} mice and significantly increased after endurance training (Tr-*Trpm4*^{+/+} mice) (Fig. 1a). In order to determine if this expression correlated with a functional TRPM4-like current in single LV myocytes, we performed electrophysiological analyses. We used caffeine (5 $\mu\text{mol/L}$) to induce an intracellular Ca^{2+} rise and to thus unmask the Ca^{2+} -activated native counterpart of the TRPM4-like current (Köster et al. 1999). The current–voltage relationship of the caffeine-induced current in isolated myocytes from Sed-*Trpm4*^{+/+} mice represents a small inward current probably due to a Ca^{2+} entry through the L-type calcium channel (Fig. 1b). In LV cardiomyocytes from Tr-*Trpm4*^{+/+}, the current–voltage relationship of the caffeine-induced current displayed an

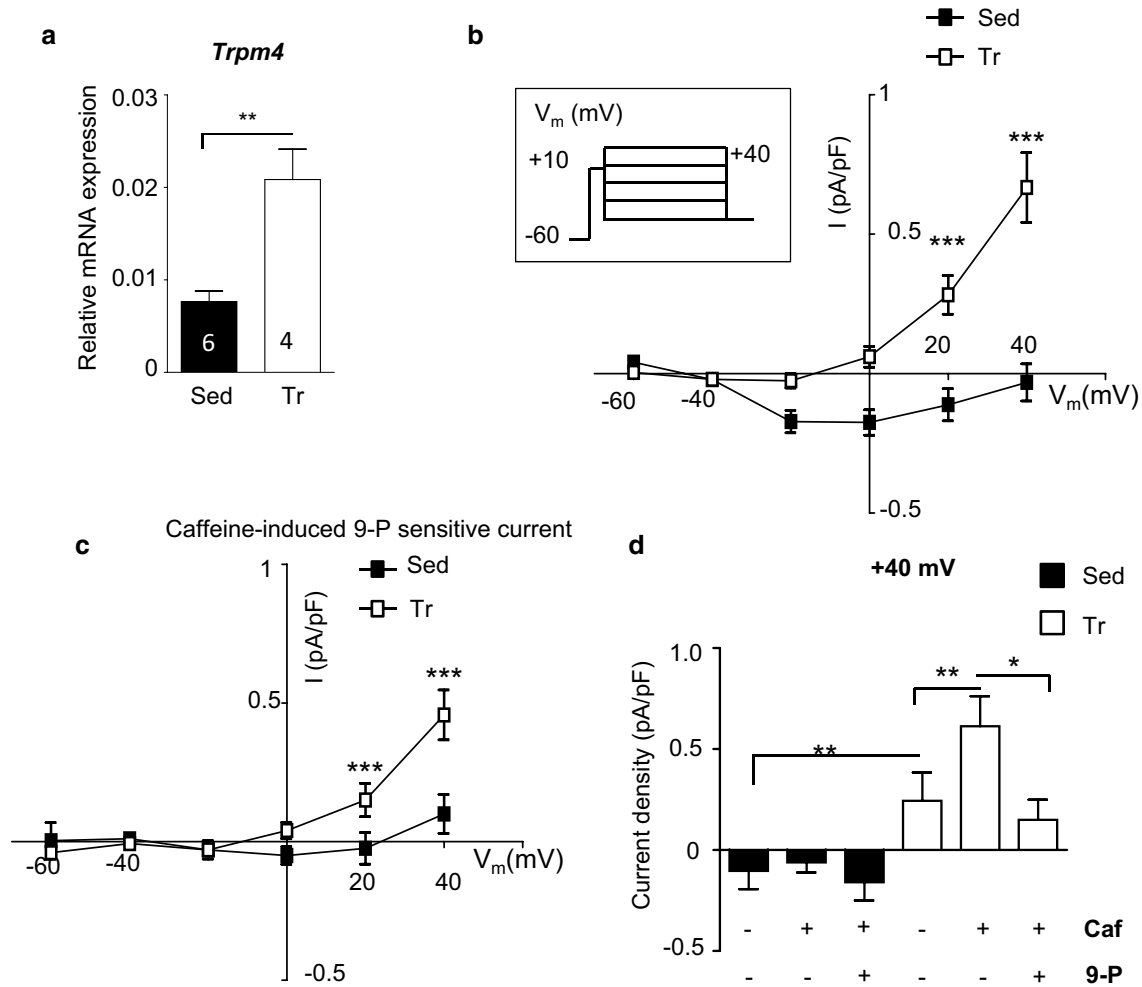


Fig. 1 Endurance training leads to TRPM4 channel functional expression in mice. **a** Relative *Trpm4* expression in the left ventricle from Sed- and Tr-*Trpm4*^{+/+} related to *Gapdh* housekeeping gene. Data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Student's *t*-test. **b** Current–voltage (*I/V*) relationships obtained in ventricular myocytes in whole cell configuration of the patch-clamp technique from Sed- (black square) and Tr- (open square) *Trpm4*^{+/+} mice. Red open circle represent the subtraction Tr–Sed. Upper panel represents the voltage clamp protocol. (Sed-

Trpm4^{+/+} *n* = 28 cells, Tr-*Trpm4*^{+/+} *n* = 21 cells). **c** *I/V* relationship of the 9-P sensitive current on the caffeine-induced current recorded on cardiomyocytes from Sed-*Trpm4*^{-/-} and Tr-*Trpm4*^{+/+} mice (*n* = 7 and 18 cells, respectively). **d** Average outward current density recorded at +40 mV in Sed- (black bar graph) and Tr- (open bar graph) *Trpm4*^{+/+} ventricular myocytes before and after caffeine (5 mM) application and with 9-Phenanthrol treatments (10 μmol/L) (*n* = 18 for each conditions). Data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Mann–Witney test was used. (Color figure online)

outward current slightly rectifying in positives voltages. The outward current was sensitive to the TRPM4 channel inhibitor 9-phenanthrol (9-P; 10 μmol/L). Indeed, the 9-P sensitive-current recorded in Tr-*Trpm4*^{+/+} cardiomyocytes also displayed an outward rectification in positive voltages and a reversal potential close to -10 mV (Fig. 1c). The 9-P application induced a 75% reduction of the current at a membrane potential of +40 mV (Fig. 1d, black histogram). By contrast, cardiomyocytes from Sed-*Trpm4*^{+/+} mice displayed a negligible sensitivity to 9-P (Fig. 1c, d). The caffeine-induced current shares the electrophysiological properties described for the TRPM4

current (Launay et al. 2002, 2004; Nilius et al. 2003, 2005; Grand et al. 2008). The rectification observed at positive voltages in the whole cell patch clamp condition is explained by a voltage dependence of the TRPM4 channel associated with an increased open probability. Both currents are 9-P sensitive. The observed reversal potential follows the pore selectivity of TRPM4 for cesium as well as lithium ions (Köster et al. 1999; Nilius et al. 2003). Our electrophysiological studies support an increase of TRPM4 channel function in LV cardiomyocytes after endurance training, consistent with the quantitative RT-PCR analysis.

Endurance training induced cellular hypertrophy but did not modify gross heart size in Tr-*Trpm4*^{-/-} mice

To clarify the potential role of TRPM4 channel expression with training, we used *Trpm4*^{-/-} mice as a negative control for the TRPM4 channel expression after training. *Trpm4*^{+/+} and *Trpm4*^{-/-} mice were separated into two groups, a sedentary group (Sed-*Trpm4*^{+/+} and Sed-*Trpm4*^{-/-} mice), which underwent only treadmill familiarization, and the trained group, which followed endurance training for 4 weeks (Tr-*Trpm4*^{+/+} and Tr-*Trpm4*^{-/-} mice). Endurance training has been described to increase cardiac function and is associated with physiologic cardiac growth via cardiomyocyte hypertrophy due to the activation of several signaling pathways (Tao et al. 2015), including the IGF-1-PI3K-Akt pathway. Training-induced cardiac remodeling was assessed in *Trpm4*^{+/+} and *Trpm4*^{-/-} groups before and after training by echocardiography. We found an increase of the left ventricle mass to body weight ratio (LV Mass/BW) in Tr-*Trpm4*^{+/+} hearts when compared to pre-training. This was associated with an increased fractional shortening and ejection fraction, demonstrating enhanced cardiac function (Table 1). This result was confirmed by an increase of whole heart to body weight ratio (Fig. 2a, lower histogram) with no modification of body weight (Fig. 2a, upper histogram). Surprisingly, we observed no changes in LV mass or functional parameters in response to training in Tr-*Trpm4*^{-/-} mice (Table 2). These results were validated by an unchanged heart weight to body weight ratio in Tr-*Trpm4*^{-/-} mice (Fig. 2c). Of note, and as previously described, *Trpm4*^{-/-} mice display left ventricle hypertrophy in basal conditions compared to *Trpm4*^{+/+} due to hyperplasia (Demion et al. 2014). As CH is commonly associated with an increase of cardiomyocytes size (Bernardo

et al. 2010), we measured cardiomyocyte surface area after endurance training. Cardiomyocytes membranes in cryosections were labeled with wheat germ agglutinin (WGA) to visualize and measure cell surface area (CSA). CSA was increased in cardiomyocytes from Tr-*Trpm4*^{+/+} mice when compared to Sed-*Trpm4*^{+/+} (Fig. 2b). Similarly, the CSA of cardiomyocytes in Tr-*Trpm4*^{-/-} mice was elevated compared to Sed-*Trpm4*^{-/-} (Fig. 2d). Measurement of cell capacitance by patch-clamp in freshly isolated cardiomyocytes confirmed the increased size of cardiomyocytes after training in both *Trpm4*^{+/+} and *Trpm4*^{-/-} mice (Fig. 2b, d, lower histogram).

Our data suggest that the gross cardiac remodeling that occurs with endurance training in *Trpm4*^{+/+} mice was absent in *Trpm4*^{-/-} mice. However, cardiomyocyte hypertrophy was comparable in trained *Trpm4*^{-/-} and *Trpm4*^{+/+} mice. We therefore hypothesized that *Trpm4*^{-/-} have an increase in cell death in response to training that could mask the heart size increase that should normally be correlated with cardiomyocyte hypertrophy.

DNA fragmentation is increased in *Trpm4*^{-/-} mice in response to endurance training

As DNA fragmentation represents a hallmark of apoptosis, we performed a TUNEL assay on heart cryosections to determine the number of TUNEL⁺ cells (Fig. 3a, b). Endurance training did not modify the number of TUNEL⁺ cells in *Trpm4*^{+/+} mice (left panels) whereas the number of TUNEL⁺ cells was increased after 4 weeks of endurance training in *Trpm4*^{-/-} mice (right panels). Death of cardiomyocytes is a characteristic of pathological CH, which normally does not occur in physiological CH. Due to this, we postulated that *Trpm4*^{-/-} mice may adapt to endurance training by using a pathological signaling pathway, instead of a physiological one.

Calcineurin but not IGF-1-PI3K-Akt is enhanced in hearts from Tr-*Trpm4*^{-/-}

We tested the two canonical hypertrophic pathways (IGF-1-PI3K-Akt and Calcineurin) by Western blot analysis in order to characterize the type of cellular hypertrophy induced by endurance training in both *Trpm4*^{+/+} and *Trpm4*^{-/-} mice. The P-Akt/Akt ratio, an indicator of IGF-1-PI3K-Akt pathway activation and physiological CH, was increased in Tr-*Trpm4*^{+/+} LV when compared to Sed-*Trpm4*^{+/+} (Fig. 4a, left panel and histogram). A recent study demonstrated that TRPM4 channel expression was associated with the chaperone protein 14-3-3 as a physical partner at the c-terminus domain (Cho et al. 2014). This chaperone protein is also activated by the IGF-1-PI3K-Akt signaling pathway (Qi et al. 2010). We found an increase

Table 1 Echocardiographic analyses induced by endurance training in *Trpm4*^{+/+} mice

Trpm4 ^{+/+}		
Parameters	Pre-training	Post-training
HR (bpm)	420 ± 20	458 ± 15
BW (g)	27.65 ± 0.69	27.60 ± 1.20
LV mass (mg)	88.35 ± 4.58	117.3 ± 4.88***
LV mass/BW (mg/g)	3.53 ± 0.18	4.69 ± 0.19***
LVEF (%)	58.96 ± 2.90	67.41 ± 3.36**
LVFS (%)	30.47 ± 2.05	37.66 ± 3.28**

Evolution of echocardiographic parameters on *Trpm4*^{+/+} 14 weeks old mice with endurance training ($n = 13 - 7$)

HR Heart rate; BW Body weight; LV mass/BW Left ventricular mass/Body weight ratio; LVEF Left ventricular ejection fraction; LVFS Left ventricular fractional shortening

Data are presented as mean ± SEM. *** $p > 0.01$, ** $p < 0.001$. Student's t -test

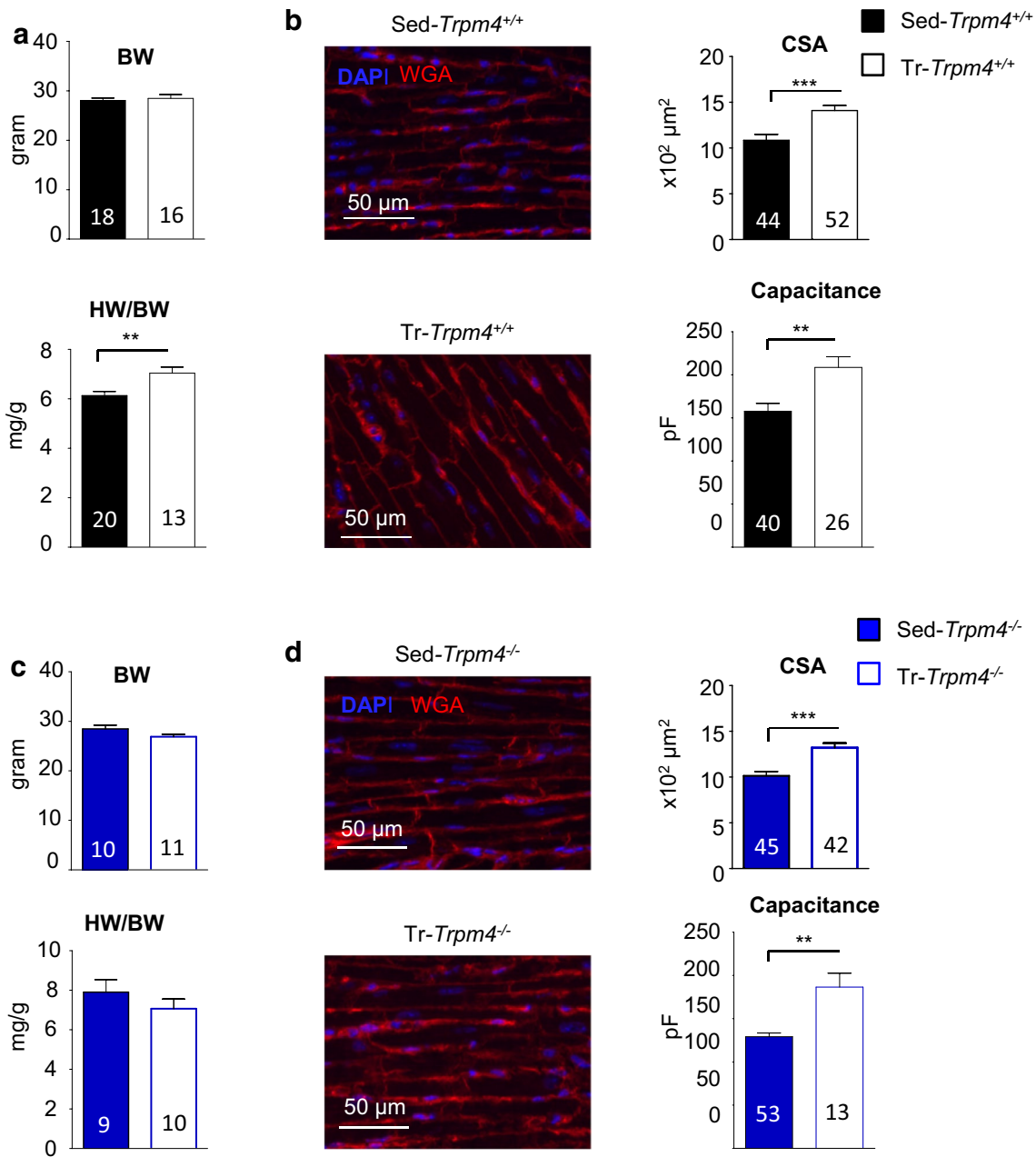


Fig. 2 Endurance training induced cardiac hypertrophy in *Trpm4*^{+/+} mice but not in *Trpm4*^{-/-} mice, while both had cardiomyocytes growth. **a** Upper histogram represents the body weight comparison between Sed and Tr-*Trpm4*^{+/+} mice. Lower histogram represents the heart weight on body weight ratio (HW/BW) comparison between Sed and Tr-*Trpm4*^{+/+} mice. **b** Cell surface labelling: in red, WGA labelling on cryosections from Sed and Tr-*Trpm4*^{+/+} mice and staining with 4,6-diamidino-2-phenylindole (DAPI) in blue. Upper histogram represents the calculated cell surface area (CSA) from longitudinal plans (Sed-*Trpm4*^{+/+} n=44 cells from six mice, Tr-*Trpm4*^{+/+} n=52 cells from seven mice). Lower histogram represents cell capacitance measurement on freshly isolated cardiomyocytes. **c** Upper

histogram represents the body weight comparison between Sed and Tr-*Trpm4*^{-/-} mice (Sed- *Trpm4*^{-/-} n=10 mice, Tr-*Trpm4*^{-/-} n=11 mice). Lower histogram represents the heart weight on body weight ratio (HW/BW) comparison between Sed and Tr-*Trpm4*^{-/-} mice. **d** In red, WGA labelling on cryosections from Sed and Tr-*Trpm4*^{-/-} mice and DAPI labeling in blue. Upper histogram represents the calculated CSA from longitudinal plans (Sed- *Trpm4*^{-/-} n=45 cells from six mice, Tr-*Trpm4*^{-/-} n=42 cells from four mice). Lower histogram represents cell capacitance measurement on freshly isolated cardiomyocytes. Data are presented as mean ± SEM. **p < 0.01, ***p < 0.001. Student's t-test

Table 2 Echocardiographic analyses induced by endurance training in *Trpm4*^{-/-} mice

<i>Trpm4</i> ^{-/-}		
Parameters	Pre-training	Post-training
HR (bpm)	431 ± 15	461 ± 19
BW (g)	27.1 ± 1.89	25.21 ± 0.63
LV mass (mg)	124.6 ± 7.71	129.4 ± 5.50
LV mass/BW (mg/g)	4.98 ± 0.30	5.17 ± 0.22
LVEF (%)	64.54 ± 3.83	63.24 ± 4.32
LVFS (%)	35.25 ± 2.64	35.09 ± 2.77

Evolution of echocardiographic parameters on *Trpm4*^{-/-} 14 weeks old mice during endurance training (*n* = 5)

HR Heart rate; *BW* Body weight, *LV mass/BW* Left ventricular mass/Body weight ratio; *LVEF* Left ventricular ejection fraction; *LVFS* Left ventricular fractional shortening

Data are presented as mean ± SEM. Student's *t*-test

of 14-3-3 protein expression in the LV from Tr-*Trpm4*^{+/+} when compared to Sed-*Trpm4*^{+/+} (Fig. 4a, middle panel and histogram), confirming activation of the IGF-1-PI3K-Akt pathway. Cn protein expression, a marker of pathological CH, was unchanged in the LV from Tr-*Trpm4*^{+/+} when compared to Sed-*Trpm4*^{+/+} (Fig. 4a, right panel and histogram). Finally, qPCR analysis of *Serca2a*, a marker of beneficial remodeling, was found to be increased in the LV from Tr-*Trpm4*^{+/+} mice when compared to Sed-*Trpm4*^{+/+} mice (Fig. 4b). Altogether, these data indicate that the cellular hypertrophy observed in response to endurance training in Tr-*Trpm4*^{+/+} mice was associated with activation of the physiological IGF-1-PI3K-Akt signaling pathway.

By contrast, the P-Akt/Akt ratio was unchanged in the LV from *Trpm4*^{-/-} mice after endurance training (Fig. 4c, left panel). The 14-3-3 protein expression was also unchanged after training (Fig. 4c, middle panel). Surprisingly, the Cn protein level was increased in Tr-*Trpm4*^{-/-} LV lysate when compared to Sed-*Trpm4*^{-/-} (Fig. 4c, right panel). The activated Cn-NFAT pathway is associated with pathological CH, heart failure and decreased *Serca2a* transcript (Molkentin et al. 1998). Validating this in our *Trpm4*^{-/-} mice, we observed a reduction of *Serca2a* mRNA expression of the LV from Tr-*Trpm4*^{-/-} mice in comparison with Sed-*Trpm4*^{-/-} (Fig. 4d). Thus, endurance training in *Trpm4*^{-/-} mice is associated with an increase in Cn expression, a hallmark of pathological CH.

TRPM4 channel regulates SOCE with endurance training

TRPM4 is a negative regulator of store-operated-Ca²⁺-entry (SOCE) in the immune system (Launay et al. 2004; Vennekens et al. 2007; Barbet et al. 2008; Serafini et al.

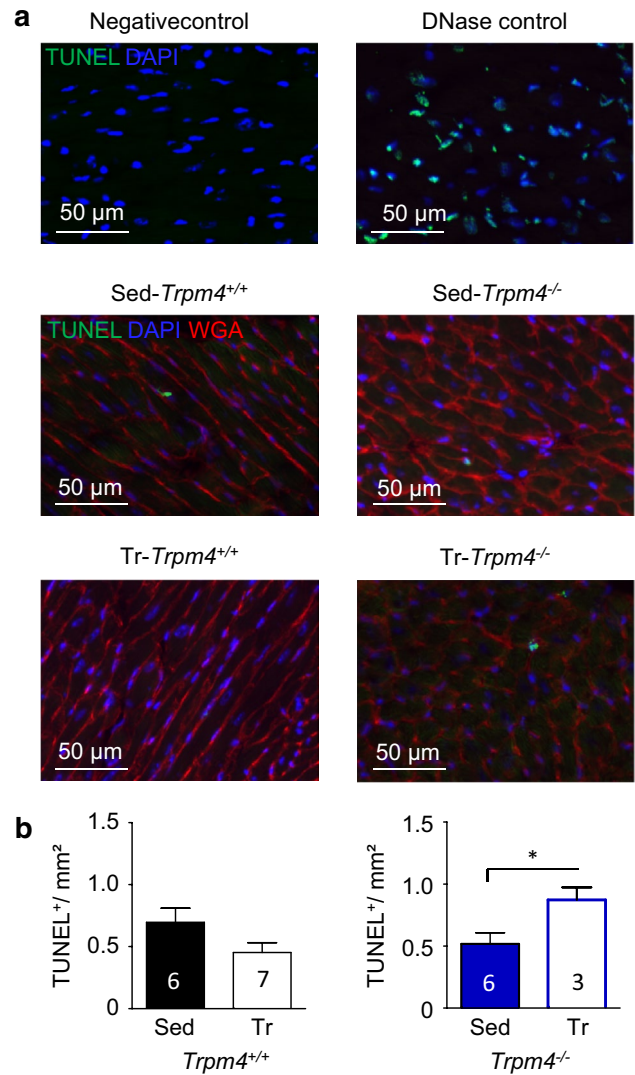


Fig. 3 Endurance training induced increased apoptosis in *Trpm4*^{-/-} mice. **a** Representative staining of apoptotic cells in cardiac sections performed with DAPI (blue) for nuclei, with WGA for the plasma membrane (red) and terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) assay (green). The images of the top are the negative and positive control, respectively. **b** The bar graphs represent the number of TUNEL⁺ nuclei per section divided by the total area (mm²). Data are presented as mean ± SEM. **p* < 0.05. Student's *t*-test. (Color figure online)

2012), as well as in cardiomyocytes (Kecskés et al. 2015), allowing a negative control of the activation of the Cn-NFAT signalling pathway in pathological hypertrophy. We investigated whether this regulation is also present in isolated LV cardiomyocytes from Sed- and Tr-*Trpm4*^{+/+} mice. After depletion of sarcoplasmic reticulum Ca²⁺ content by caffeine (5 mmol/L) in a Ca²⁺-free Tyrode solution, SOCE was measured following the addition of 1 mmol/L of free Ca²⁺. SOCE was similar between isolated LV cardiomyocytes from Sed-*Trpm4*^{+/+} and Tr-*Trpm4*^{+/+} mice (Fig. 5a).

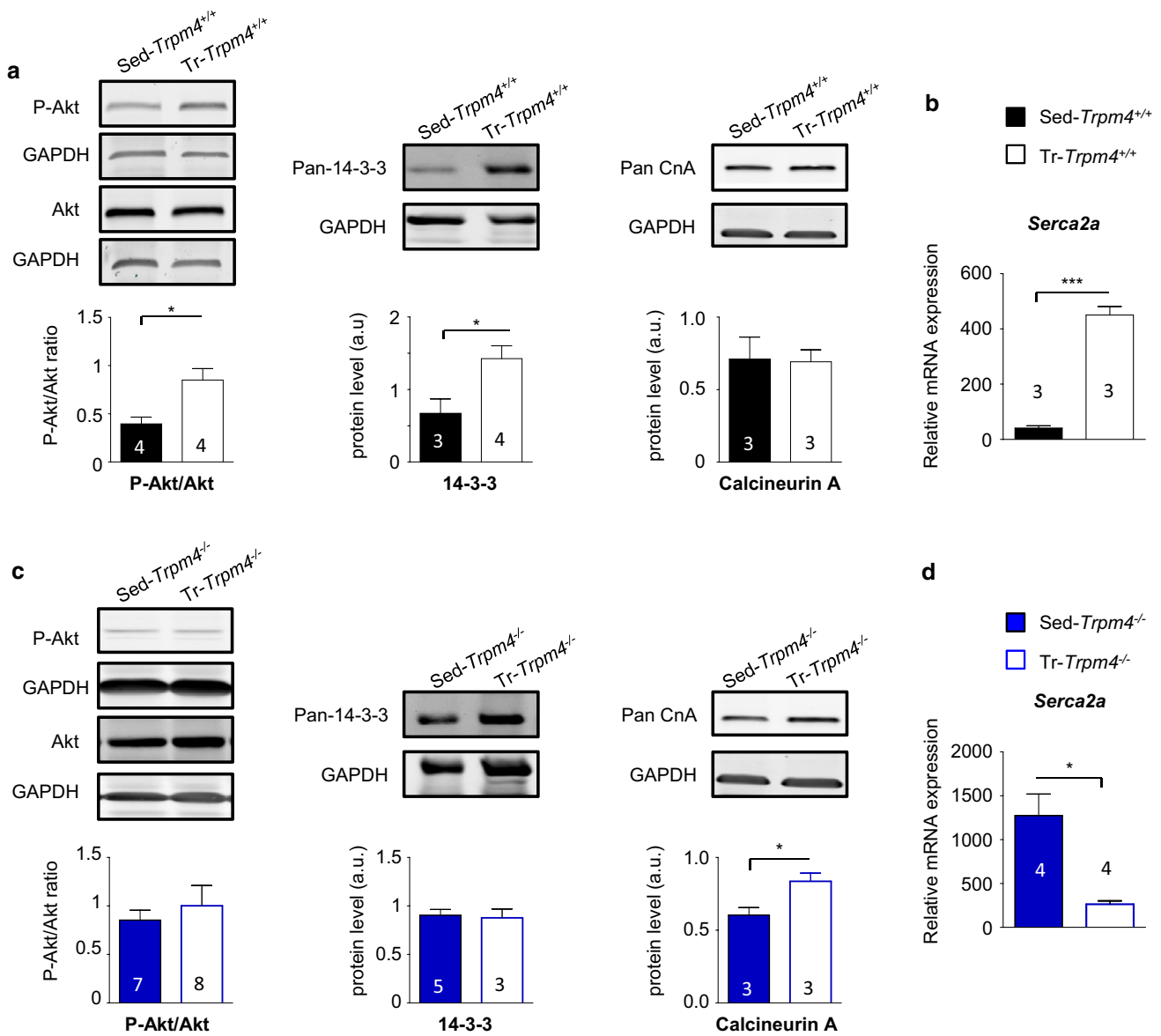


Fig. 4 Characterization of the pro-hypertrophic pathway activated by endurance training in *Trpm4*^{+/+} and *Trpm4*^{-/-} mice. **a** Relative P-Akt/Akt ratio, Pan-14.3.3 and Pan CnA proteins expression assessed by Western-blot analysis normalized to loading control GAPDH. **b** Relative expression of *Serca2a* mRNA assessed by quantitative PCR. Data are presented as mean ± SEM. **p* < 0.05, ****p* < 0.001. Student's *t*-test

c Relative P-Akt/Akt ratio, Pan-14.3.3 and Pan Cn proteins expression assessed by Western-blot analysis normalized to loading control GAPDH. **d** Relative expression of *Serca2a* mRNA assessed by quantitative PCR. Data are presented as mean ± SEM. **p* < 0.05, ****p* < 0.001. Student's *t*-test

To detect whether TRPM4 channel is involved in the regulation of calcium entry as was demonstrated in the immune system, we pre-incubate the cardiomyocytes with 10 μM 9-P during SOCE measurement (Vennekens et al. 2007; Barbet et al. 2008; Serafini et al. 2012). Pre-incubation with 9-P (Fig. 5b) increased SOCE in LV myocytes from Tr-*Trpm4*^{+/+} mice only and not from Sed-*Trpm4*^{+/+} mice. Calculation of the area under the curve (AUC) demonstrated the significance of this observation (Fig. 5c). These results suggest that the TRPM4 channel is open during SOCE. Increased SOCE in the presence of 9-P suggests that the

TRPM4 channel, when opened, negatively regulates Ca²⁺ entry. Altogether, this data suggests a potential involvement of the TRPM4 channel in CH adaptations via the regulation of Ca²⁺ entry.

SOCE was also measured in isolated cardiomyocytes from Sed-*Trpm4*^{-/-} and Tr-*Trpm4*^{-/-} mice. No changes in SOCE amplitude were identified between basal conditions and 9-P treatment in isolated myocytes from either Sed-*Trpm4*^{-/-} or Tr-*Trpm4*^{-/-} mice (Fig. 5d, e). The absence of a difference in AUC calculations (Fig. 5f) reinforces the observation that 9-P unmasked the TRPM4

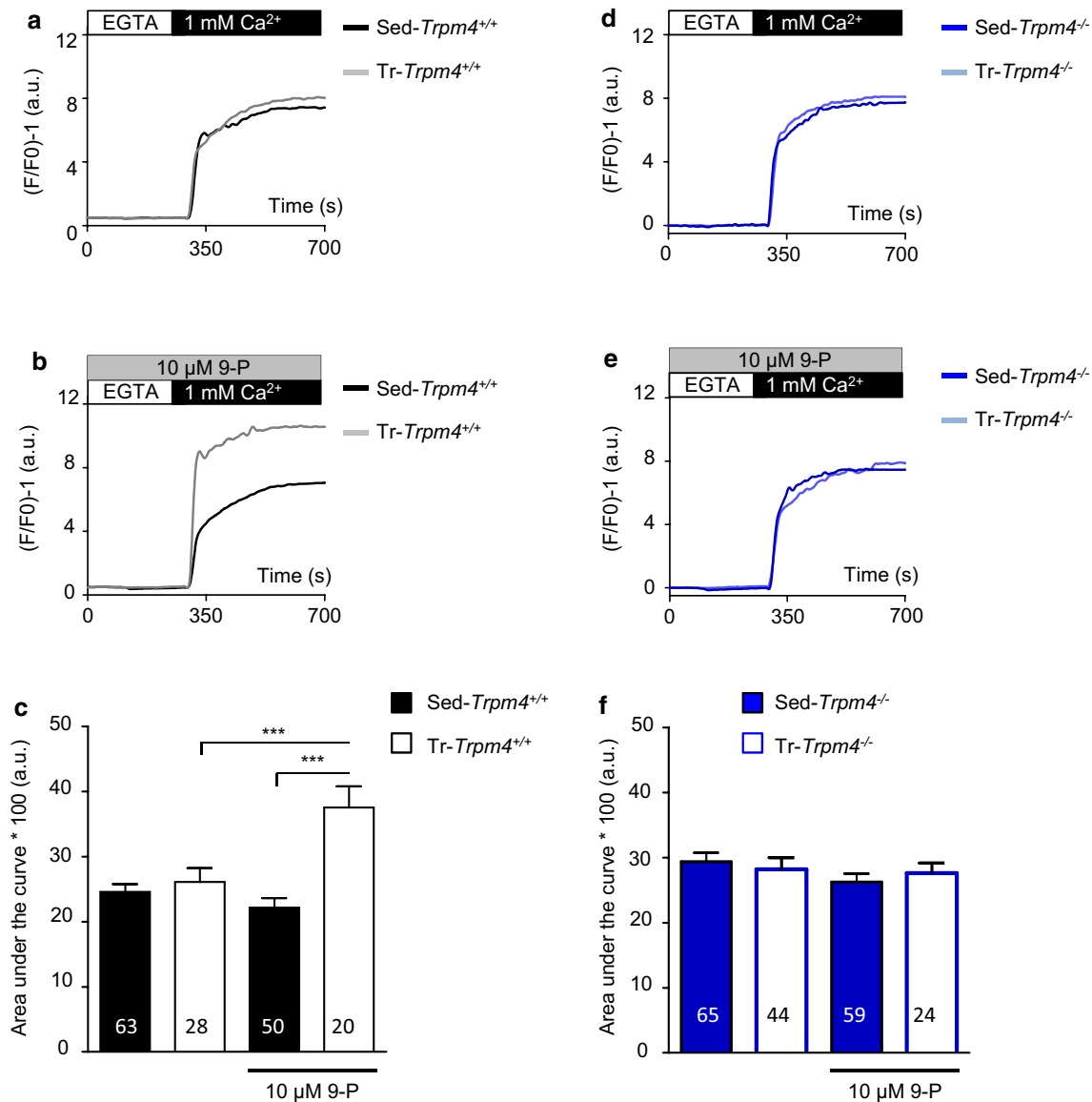


Fig. 5 Store-operated calcium entry measurement in freshly isolated cardiomyocytes from *Trpm4*^{+/+} and *Trpm4*^{-/-} mice **a** Mean fluorescence evolution during the application of 1 mM free Ca²⁺ solution in Sed (in black) and Tr-*Trpm4*^{+/+} (in grey) freshly isolated myocytes. **b** Same experience as in **a**. In presence of 9-Phenanthrol (9P) in Sed and Tr-*Trpm4*^{-/-} freshly isolated myocytes. **c** The calculated areas under the curve after calcium application from single cell recording

are presented in histograms. **d** Mean fluorescence evolution during the application of 1 mM free Ca²⁺ solution in Sed (in black) and Tr-*Trpm4*^{-/-} (in grey) freshly isolated myocytes. **e** Same experience as in **d**. in presence of 9P in Sed and Tr-*Trpm4*^{-/-} freshly isolated myocytes. **f** The calculated areas under the curve after calcium application from single cell recording are presented in histograms. Data are presented as mean ± SEM. ****p* < 0.001. Two-way ANOVA analysis

functional expression in Tr-*Trpm4*^{+/+} mice and strengthened the SOCE negative regulation by TRPM4 channel in cardiomyocytes.

Discussion

In this study, as expected, *Trpm4*^{+/+} mice displayed increased LV heart muscle mass associated with cardiomyocyte hypertrophy in response to endurance training. We

confirmed activation of the pro-survival PI3K-Akt signal transduction pathway in the hearts of Tr-*Trpm4*^{+/+} mice by an increased P-Akt/Akt ratio, the branch point of the physiological CH signaling cascade (Kemi et al. 2008; Bernardo et al. 2010). We revealed a concomitant increase of the functional expression of the TRPM4 channel after 4 weeks of endurance training in *Trpm4*^{+/+} mice that was not evident in sedentary mice. When incubated in the presence of 9-P, a TRPM4 channel inhibitor, cardiomyocytes from Tr-*Trpm4*^{+/+} mice exhibited an increased Ca²⁺-entry through

store-operated Ca^{2+} channels, consistent with the negative regulation of SOCE by the TRPM4 channel. By contrast, in the absence of TRPM4 in *Trpm4^{-/-}* mice, no change on SOCE was detected with or without 9-P incubation, validating the direct involvement of TRPM4 channel activity in cardiomyocytes from *Tr-Trpm4^{+/+}* mice.

Surprisingly, in the TRPM4 channel deficient mice, despite their basal hypertrophy due to an increased number of cardiomyocytes (Demion et al. 2014), we did not observe an increase of heart size or functional modifications in response to training while cardiomyocytes hypertrophied. We hypothesized that this phenomenon could be related to an increased number of apoptotic cells. Apoptosis, or programmed cell death, when increased, is an indicator of heart failure (Cho et al. 2016). In *Tr-Trpm4^{-/-}* mice, the increase of TUNEL⁺ nuclei, a marker of apoptotic cells, correlated with the activation of the pathological CH Cn-NFAT pathway without any modifications of PI3K-Akt pathway. Altogether, these data suggest that the IGF-1-PI3K-Akt pathway activation by endurance training positively correlates with TRPM4 channel functional expression. This

functional expression could, in turn, contribute to the negative control of Ca^{2+} -entry and thus prevent the pathological, pro-hypertrophic Ca^{2+} -dependent Cn-NFAT signaling pathway (Fig. 6). Consistent with our data, it was recently demonstrated that TRPM4 channel regulates the pathological hypertrophy induced by Angiotensin II infusion (Kecskés et al. 2015). Specifically, transgenic mice with a cardiac specific *Trpm4* gene deletion displayed a worsened CH associated with an augmented activation of the Cn-NFAT signaling pathway (Kecskés et al. 2015).

TRPM4 channel belongs to the TRPM (transient receptor potential melastatin) channel family. This heterogeneous family includes ionic channels involved in melanoma aggressiveness (TRPM1) (Dalal et al. 2013) to channels involved in menthol and cold sensation (TRPM8) (Palkar et al. 2015). TRPM6 and TRPM7 are very particular members of the family due to a kinase domain capable of phosphorylating itself and other substrates (Runnels et al. 2001; Runnels 2011). While most of TRP channels are calcium permeable, TRPM4 and TRPM5 channels are the exception by their ability to be

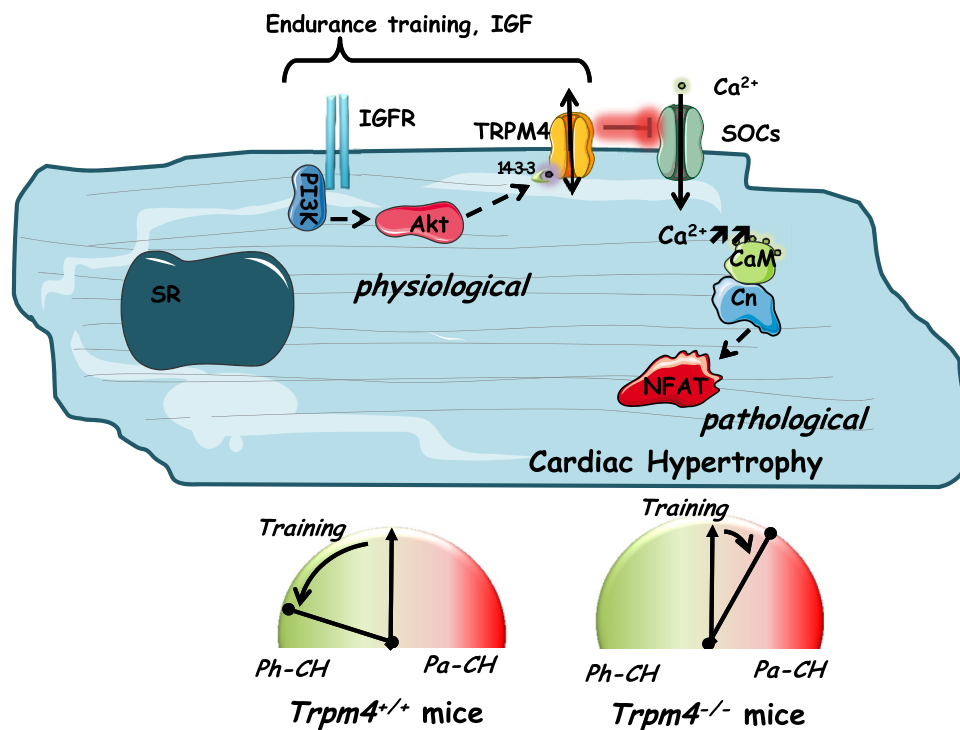


Fig. 6 Proposed model of TRPM4 channel role in CH signalling. The IGF-1-PI3K-Akt pathway activation by endurance training induces the activation of the chaperone protein 14-3-3. This chaperone protein may induce increased expression of TRPM4 channel at the plasma membrane. Once expressed at the membrane, TRPM4 channel can negatively regulate SOCE and thus the Cn-NFAT pathway. This may be beneficial for Ph-CH as well as Pa-CH remodelling due to a favouring of the IGF-1-PI3K-Akt pathway. When TRPM4 channel expression is blunted, there is no inhibitory regulation of Cn-NFAT

pathway. Due to this, the IGF-1-PI3K-Akt pathway is counterbalanced by the Cn-NFAT pathway, leading to no adaptations to training. Abbreviations: *SR* sarcoplasmic reticulum; *IGF* insulin growth factor; *IGFR* IGF receptor; *Et-1* endothelin-1; *PI3K* phosphoinositide 3 kinase; *SOCs* store operated channels; *SACs* stretch activated channels; *ROC*s receptor operated channels; *CaM* calmodulin; *Cn* calcineurin; *NFAT* nuclear factor of activated T cell; *PMI* post-myocardial infarction

selective to sodium and potassium but not calcium (Ullrich et al. 2005). Although TRPM4 and TRPM5 channels have similar actions, they do not share the same pattern of expression and cannot compensate each other's expression (Barbet et al. 2008). While it was demonstrated that TRPM7 channel was important in the cardiogenesis and automaticity (Sah et al. 2013a, b), TRPM6 was involved in atrial fibrillation (Zhang et al. 2015). TRPM4 channel is the most studied channel in the heart function, particularly in conduction disorders (Liu et al. 2010, 2013; Simard et al. 2012, 2013; Abriel et al. 2012; Mathar et al. 2014; Jacobs et al. 2015; Hof et al. 2016).

TRPM4 channel is known to negatively regulate SOCE in several cell types including non-excitabile and excitable cells, but also in heterologous expression systems (Lau-nay et al. 2004; Vennekens et al. 2007; Barbet et al. 2008; Serafini et al. 2012; Kecskés et al. 2015). For example, single ventricular cardiomyocytes from cardiac-specific TRPM4 KO mice displayed a significant increase of SOCE when compared to cardiomyocytes from WT mice after angiotensin II treatment (Kecskés et al. 2015). In line with this, in the present study, using 9-P, we unmasked a Ca^{2+} entry regulation by TRPM4 activity in cardiomyocytes from *Tr-Trpm4^{+/+}* mice. This regulation is absent in cardiomyocytes from *Tr-Trpm4^{-/-}*. These results, in combination with previous studies, demonstrate that the TRPM4 channel negatively regulates Ca^{2+} entry in hypertrophied cardiomyocytes. By regulating Ca^{2+} -entry into the cardiomyocytes, TRPM4 channel might thus be able to regulate the Ca^{2+} -dependent Cn-NFAT activation pathway.

In hearts from patients with heart failure (HF) and presenting CH, Cn activity is increased (Haq et al. 2001; Die-drichs et al. 2004). Regular physical activity can improve the quality of life in patients with chronic HF and reverse pathological cardiac remodeling. In mice presenting HF with CH and activated Cn-NFAT signaling pathway, exercise training can prevent the activation of the pathological CH Cn-NFAT pathway (Oliveira et al. 2009). Consistently, we demonstrate here that, concomitant to TRPM4 channel functional expression, the physiological cardiac hypertrophy signaling pathway IGF-1-PI3K-Akt is favored by endurance training, whereas Cn expression remains unchanged. The opposite results were identified when TRPM4 is absent, an elevated Cn expression without enhanced Akt phosphorylation. A balance between these CH signaling pathways similar to that in the current study has been reported previously. In cultured cardiomyocytes from rat, endothelin-1 induced hypertrophy was inhibited by the peroxisome proliferator activated receptor alpha (PPAR- α) activation via Akt phosphorylation and NFAT nuclear translocation prevention (Li et al. 2007). These evidences suggest that a counter balance may exist in the regulation of these signaling pathways.

We also correlated an increased expression of the 14-3-3 protein with Akt activation after training. The 14-3-3 protein is a chaperone protein, activated by the Akt pathway, and involved in the response to IGF-1. 14-3-3 recognizes and conducts Serine/threonine phosphorylated proteins to their target destination. A recent study demonstrated that a physical interaction exists between the 14-3-3 protein and the TRPM4 channel protein in the brain (Cho et al. 2014). Our data suggest that during endurance training, the activation of Akt by phosphorylation is associated with an increase of the 14-3-3 protein expression (Qi et al. 2010). This increased 14-3-3 protein may in turn favor TRPM4 channel expression at the plasma membrane and thus its function (Cho et al. 2014). Finally, this improved TRPM4 channel functional expression may negatively regulate Ca^{2+} -entry through the plasma membrane and thus the activation of the Cn-NFAT pathway. However, in the absence of TRPM4 channel expression during endurance training, cellular hypertrophy pairs with elevated Cn expression, suggesting that TRPM4 expression may be linked to the physiological CH signaling pathway.

Additional studies have correlated the Akt pathway to TRP channel function. Indeed, it was demonstrated that in the absence of TRPM4 channel in macrophages, impaired calcium mobilization and down-regulated Akt signaling pathway were correlated (Serafini et al. 2012). It was also shown that TRPC channels are involved in the IGF1R-PI3K-Akt pathway via calcium signal in epithelial cells (Dai et al. 2014). Moreover, PI3K pathway activation was shown to favor TRPC6 expression by increasing its exocytosis (Xie et al. 2012).

In summary, these data suggest that TRPM4 expression may be protective by correlating with the pro-survival effects of the IGF-1-PI3K-Akt signal transduction pathway. This may in turn involve a regulatory effect of enhanced 14.3.3 protein expression in association with the TRPM4 channel. In addition, these finding further support a negative regulation of TRPM4 in the Cn pathway. In conclusion, these findings strengthen the importance of the TRPM4 channel in the cardiac physiopathology.

Materials and methods

All procedures conformed to the European parliament directive 2010/63/EU and were approved by the local institutional committee (CEEA-LR-12110).

Animals

Knock-out mice (*Trpm4^{-/-}*) and wild-type littermate controls (*Trpm4^{+/+}*) obtained as previously described (Barbet et al. 2008; Demion et al. 2014) were randomly

separated into four groups: Sedentary-*Trpm4*^{+/+} (Sed-*Trpm4*^{+/+}), Trained-*Trpm4*^{+/+} (Tr-*Trpm4*^{+/+}), Sedentary-*Trpm4*^{-/-} (Sed-*Trpm4*^{-/-}) and Trained-*Trpm4*^{-/-} (Tr-*Trpm4*^{-/-}). Mice were sacrificed at 14 weeks of age by cervical dislocation.

Exercise training protocol

Trpm4^{+/+} and *Trpm4*^{-/-} mice were assigned randomly to the sedentary or trained group. Sedentary mice at 12 weeks old were subjected to 2 weeks of familiarization on the rodent treadmill (15 min at 12 m/min, 5% incline, 5 days/week) and with the experimenter. Trained mice, at 8 weeks old, were subjected to the same 2 weeks of treadmill familiarization, followed an endurance training of 4 weeks (70% of their maximal aerobic speed, from 30 min/day to 75 min/day with a 15 min increase /week, 5 days/week, 5% incline). Before the beginning of the 4 weeks endurance training, all mice underwent an exhaustive exercise to determine maximal aerobic speed of each individual mouse. Briefly, exhaustive exercise consisted of: 10 min at 10 m/min, followed by increased treadmill speed by 2 m/min every 2 min until exhaustion and fatigue of the mouse. Exhaustion was defined as the mouse's inability to maintain running and 5×5 sec on the electrical grid stimulus. The speed at exhaustion was recorded as their maximal aerobic speed (Supplementary Fig. 1).

Echocardiography

Trpm4^{+/+} and *Trpm4*^{-/-} mice were anesthetized with isoflurane and echocardiography was performed using Vevo 2100 ultrasound system (VisualSonics, Toronto, Canada) equipped with a real-time micro-visualization scan head probe (MS-550D) operating at a frame rate ranging from 740 frames per sec and a nosepiece-transducer used with a central frequency of 22–50 MHz, as previously described (Demion et al. 2014). All measurements and calculations were performed with the Vevo 2100 Software and were averaged from a minimum of four cycles and followed the standards of the American society of echocardiology and the Vevo 2100 protocol-based measurements and calculations guidelines. Echocardiography was performed in each trained animal at the end of the treadmill familiarization and following 4 weeks of endurance training. Before the beginning of the 4 weeks endurance training, to determine maximal aerobic speed of mice, all mice underwent an exhaustive exercise. Briefly, exhaustive exercise consists in: after 10 min at 10 m/min, treadmill speed was increased by 2 m/min every 2 min until exhaustion and fatigue of the mouse. Exhaustion was defined as the mouse inability to maintain running and 5×5 sec on the electrical grid

stimulus. The speed of exhaustion was recorded as their maximal aerobic speed (Supplementary Fig. 1).

Cardiomyocytes isolation

The mouse was euthanized by cervical dislocation, after which the heart was rapidly excised and retrogradely perfused (Langendorff) at 37 °C for 6–8 min with a modified Tyrode solution (in mmol/L: NaCl 113; KCL 4.7; KH₂PO₄ 0.6; Na₂HPO₄ 0.6; MgSO₄ 1.2; NaHCO₃ 12; KHCO₃ 10; HEPES 10; Taurine 30; pH 7.4) containing 0.1 g/mL liberase dispase high research grade (Roche, France). Cells were kept in 1.8 mmol/L Ca²⁺ (20–22 °C) before starting recordings experiments.

Patch clamp recordings

Whole-cell patch-clamp recordings were performed (Axon Instruments, Foster City, CA) at room temperature (22–24 °C) to record cell membrane capacitance in LV myocytes enzymatically isolated from *Trpm4*^{+/+} and *Trpm4*^{-/-} mice. Cell capacitance was measured following brief voltage steps (± 10 mV) from the holding potential to provide an estimation of cell size.

TRPM4-like current recording were performed using the whole cell configuration of the patch-clamp technique using an Axopatch 200B (Axon instruments). Extracellular solution contained (mmol/L): 140 LiCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, 20 tetraethylammonium chloride (TEA) buffered at pH 7.4 with LiOH. Pipette solution contained (mmol/L): 140 CsCl, 20 TEA-Cl, 1 MgCl₂, 10 HEPES and 10 EGTA, pH 7.2 with CsOH. The Ca²⁺ concentration in the pipette was adjusted to 60 nmol/L by adding appropriate amounts of CaCl₂ calculated by the CaBuf program. Patch electrodes had a DC resistance between 2 and 4 M Ω when filled with the different recording solutions. Currents were sampled at 2.5 kHz and filtered at 10 kHz. The protocol consisted of test pulses which were preceded by five depolarizing prepulses from –60 to +10 mV in order to load the SR with Ca²⁺ to a similar extent, followed by 5 s pulse from –40 to 40 mV with 20 mV ΔV_m . Data were analyzed using the pClamp 10.0 software (molecular devices corporation).

Calcium measurements

Changes in intracellular Ca²⁺ concentration [(Ca²⁺)_i] were determined in ventricular cardiomyocytes using the ratiometric fluorescent Ca²⁺ indicator Fura-2 probe. Cells were loaded in MaTteck by incubation with 2.5 μ M Fura-2AM (Tef-labs) plus 0.02% pluronic acid F-127 (Molecular Probes Inc) in Ringer EGTA solution (in mM: 145 NaCl, 5.4 KCl, 1 MgCl₂, 10 glucose, 10 N-(hydroxyethyl)

piperazine-*N'*-2-ethanesulfonic acid, 5 mM EGTA; pH was adjusted to 7.5 with NaOH) during 30 min at 37 °C in a humidified air atmosphere. The loaded cells were then washed and placed with Ringer EGTA solution without Ca²⁺ and containing Nifedipine 10 μM and KB-R7943 5 μM and mounted on the microscope stage at room temperature for 15 min to allow Fura-2AM de-esterification. Measurements of the Fura-2 probe fluorescence ratio were made from images captured digitally, every second, by a cooled CCD camera (Photometrics) with a X20 objective lens mounted on an inverted microscope (Axiovert, Zeiss), and acquired after excitations at 340- and 380 nm using a lambda-DG4 excitation system (Sutter Instrument Company, USA). Ca²⁺ signals were then analyzed by using the Metafluor software (Universal Imaging Corporation, USA). The results are given as the variation from basal values of the ratio of 340/380 nm recordings with fluorescence values corrected for background and dark currents.

Western blot analysis

Frozen left ventricle (LV) tissue (≈50 mg) was homogenized using a manual polytron instrument. LV tissue was lysed under rotating agitation during 45 min in an extraction buffer containing protease and phosphatase inhibitors (Roche). After 5 min of centrifugation at 10,000 g at 4 °C, proteins were collected from the supernatant. Protein expression was assessed by immunoblot analysis with the primary antibodies anti-PhosphoSer473-Akt (P-Akt; #4060), anti-Akt (#9171), Pan-CalcineurinA (#2614) and Pan 14-3-3 (#8312) (Cell Signaling, Ozyme, Montigny-le-Bretonneux, France). Each protein was quantified using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (PA1-913, Fisher Scientific, Illkirch, France) as a loading control with ImageJ software. All immunoblots were visualized using the Odyssey[®] infrared imaging system (LI-COR Biosciences, Lincoln, Nebraska, USA) coupled to infrared-labeled anti-goat, anti-mouse or anti-rabbit IgG secondary antibodies (1/30,000 dilution) (LI-COR Biosciences, Courtaboeuf, France).

TUNEL assay method

Apoptotic nuclei were detected in heart sections by in situ terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-conjugated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL), with the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) in accordance with manufacturer recommendations. The total number of TUNEL-positive cardiomyocytes, indicated by a green nucleus, per section was quantified. dUTP labelling was not observed when TdT was omitted from the reaction (negative control). The number of TUNEL⁺ cells was

determined as the number of labeled positive cardiomyocytes per section divided by the total area (mm²).

Wheat germ agglutinin labelling

Adult heart 10 μm cryosections were stained with wheat germ agglutinin (WGA 1:250; Vector Labs) counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000 from Sigma–Aldrich), and examined using AxioVision Rel.4.8 software.

PCR

Genomic PCR was performed on tail DNA with primers (Eurofin genomics, Ebersberg, Deutschland) specific for the wild-type and null alleles as described before (Barbet et al. 2008). Total RNA was isolated from a minimum of five samples per group using a Nucleospin total RNA isolation kit (Macherey–Nagel, Hoerd, France) according to the manufacturer's instructions. Total RNA, oligo-dT and random hexamer primers were used to generate cDNA using a Verso enzyme kit (ThermoFischer, Illkirch, France). RT-PCR for the evaluation of the expression of *Trpm4*, *Serca2a* and *Gapdh* was performed using gene-specific primers and performed in duplicate. Reactions were achieved using SYBR green Mix (Roche Applied System, Meylan, France) and commercially prepared primers (Sigma-Aldrich, Saint-Quentin Fallavier, France) (see primer sequences in Table 1).

Primer	Forward	Reverse
<i>mTrpm4</i>	CCAGGGCTCATTCCC CGCAAGGT	ATTCAC CTGCCT CTTCCT CCCG
<i>mSerca2a</i>	AGTTCATCCGCTACC TCATCTCA	CACCAG ATTGAC CCAGAG TAACTG

Statistics

All statistical analyses and artworks were performed using Prism (GraphPad) software. Data are expressed as mean ± SEM. Differences between groups were compared by two-sample *t* test and two-way repeated measurement ANOVA. When assumptions were not valid, non-parametric tests were performed (Mann–Whitney test). For comparison of echocardiographic parameters recorded in the same mice before and after training, called pre- versus post-training respectively, a paired *t*-test was used. *P*-values of 0.05 or less indicated significant difference between groups. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *ns* no significant.

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