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Upregulation of transient receptor potential melastatin 4 (TRPM4) in ventricular fibroblasts from heart failure patients

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Abstract

The transient receptor potential melastatin 4 (TRPM4) is a Ca^{2+}-activated nonselective monovalent cation channel belonging to the TRP channel superfamily. TRPM4 is widely expressed in various tissues and most abundantly expressed in the heart. TRPM4 plays a critical role in cardiac conduction. Patients carrying a gain-of-function or loss-of-function mutation of TRPM4 display impaired cardiac conduction. Knockout or over-expression of TRPM4 in mice recapitulates conduction defects in patients. Moreover, recent studies have indicated that TRPM4 plays a role in hypertrophy and heart failure. Whereas the role of TRPM4 mediated by cardiac myocytes has been well investigated, little is known about TRPM4 and its role in cardiac fibroblasts. Here we show that in human left ventricular fibroblasts, TRPM4 exhibits typical Ca^{2+}-activation characteristics, linear current–voltage (I–V) relation, and monovalent permeability. TRPM4 currents recorded in fibroblasts from heart failure patients (HF) are more than 2-fold bigger than those from control individuals (CTL). The enhanced functional TRPM4 in HF is not resulted from changed channel properties, as TRPM4 currents from both HF and CTL fibroblasts demonstrate similar sensitivity to intracellular calcium activation and extracellular 9-phenanthrol (9-phen) blockade. Consistent with enhanced TRPM4 activity, the protein level of TRPM4 is about 2-fold higher in HF than that of CTL hearts. Moreover, TRPM4 current in CTL fibroblasts is increased after 24 hours of TGFβ1 treatment, implying that TRPM4 *in vivo* may be upregulated by fibrogenesis promotor TGFβ1. The upregulated TRPM4 in HF fibroblasts suggests that TRPM4 may play a role in cardiac fibrogenesis under various pathological conditions.

Keywords

TRP channels; TRPM4; Calcium signaling; Human ventricular fibroblasts; Heart failure

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Introduction

TRPM4 is a calcium activated nonselective cation channel [43, 44] belonging to the melastatin subfamily of transient receptor potential (TRP) membrane protein superfamily [9, 49, 50]. Among the 28 mammalian TRP channels, TRPM4 and its close homologue, TRPM5, are the only two members which are activated by a rise of intracellular Ca^{2+} but are calcium-impermeable monovalent cation channels [44, 56, 35]. The channel function of TRPM4 is regulated by PIP_2 [75], voltage [51, 35], hypotonic cell swelling [20], decavanadate [52], intracellular nucleotides such as ATP, and polyamines [53]. Recent cryo-EM structure studies have revealed mechanistic insights of monovalent permeability; binding domains for ATP, PIP_2, and Ca^{2+}; and how the channel gating can be regulated [1, 18, 70, 30]. Moreover, TRPM4 channel protein is post-translational modified by SUMOylation [42, 46], phosphorylation [11], and glycosylation [67].

TRPM4 is widely expressed in many different types of cells and tissues, and appears to be involved in various physiological functions, including smooth muscle function [19], insulin secretion [8, 62], immune response [68, 61, 2], neuronal degeneration [60], ischemic stroke [7, 71], and cardiovascular functions [41]. TRPM4 is most abundantly expressed in the heart [43, 44]. A gain-of-function mutation at the N-terminal (E7K) of TRPM4 was first found to be associated with progressive familial heart block type I (PFHBI) [42]. A large number of other mutations of TRPM4 were later identified to be associated with PFHBI [12], isolated cardiac conduction block (ICCD) [46, 65], atria-ventricular conduction block (AVB) [65, 66], right bundle branch block (RBBB) [65], bradycardia [45], Brugada syndrome (BrS) [45, 26], and complete heart block (CHB) [4]. Among these mutants, gain- or loss-of-function of TRPM4 seems to be mainly caused by changes of membrane protein level of TRPM4 [4, 41]. Consistent with conduction block in TRPM4 mutation–carrying patients, knockout of TRPM4 or over-expression of TRPM4 in mice results in cardiac arrhythmias [47, 58, 63].

TRPM4 not only plays an essential role in cardiac conduction [27] but also seems to be involved in hypertension, hypertrophy, and heart failure. $TRPM4^{-/-}$ mice are hypertensive [48] and display increased β-adrenergic inotrophy in the ventricular myocardium [47], as TRPM4 deletion causes elevated circulating catecholamine levels due to increased acetylcholine-induced exocytosis in chromaffin cells [48]. TRPM4 deletion mice also exhibit enhanced response to angiotensin II–induced cardiac hypertrophy [40], and left ventricular (LV) eccentric hypertrophy at an older age (32 weeks) [14]. Deletion of TRPM4 in rats aggravates right ventricular hypertrophy induced by right ventricular pressure load [24]. Whereas TRPM4 deficiency appears to exacerbate hypertensive hypertrophy and heart failure, controversial results have been reported about the role of TRPM4 in ischemic heart failure [32, 38, 57]. It was previously shown that TRPM4 knockout improves survival rate and enhances β-adrenergic cardiac reserve after inducing ischemic heart failure [38], and TRPM4 inhibitor 9-phen was also found to reduce ischemia–reperfusion-induced cardiac death in rats [57]. However, a recent study demonstrated that TRPM4 is required for mice to survive in ischemic heart failure [32].

Whereas the function of TRPM4 mediated by cardiac myocytes have been extensively investigated, little is known about TRPM4 in cardiac fibroblasts, the most abundant cells

in the heart, which are involved in various pathological processes in heart diseases. A recent study reported functional expression of TRPM4 and its potential role in human atrial fibroblast growth in culture [64]. Our previous study also showed that TRPM4 expression in mouse fibroblasts can be potentiated by fibrosis-promoting factor TGFβ1 [17]. These data imply that TRPM4 may influences fibroblasts' functions in the heart. In the present study, we demonstrate that TRPM4 is functionally expressed in human ventricular fibroblasts and is markedly enhanced in heart failure patients. Our results suggest that TRPM4 may play a role in cardiac fibrogenesis cascade under pathological conditions.

Materials and methods

Tissue freezing and fibroblast isolation from failing and nonfailing human left ventricular tissues

Human left ventricular tissues were obtained from donor hearts (Table 1) provided by Illinois Gift of Hope (GOH) Organ & Tissue DonorNetwork. These studies were approved by the Human Study Committees of Rush University Medical Center and Illinois GOH. Consent was obtained by GOH from the donors' families for the use of the donor hearts for research purposes, and studies were performed in accordance with the Declaration of Helsinki. The failing hearts were from the organ donors who had a history of heart failure with an ejection fractioning (EF) of 14.5±2.5 (n=6), reflecting a significantly impaired cardiac function in these failing human hearts (Table 1). The nonfailing control hearts (CTL) were from the donors who had a normal cardiac function with the EF of 62.2±3.6% (n=6) and without a history of any major cardiovascular disease. Those well-procured human hearts were obtained by the Ai lab through the GOH. Following heart explanation, left ventricular tissues were quickly dissected to be flash frozen for western blot (WB) experiments, or to be used immediately for isolation of fibroblasts for functional studies.

Fibroblasts were isolated in the Ai lab using the method as we previously described [17]. In brief, left ventricles of CTL or HF heart samples were minced and incubated with collagenase (150~200 U/ml CLS II, Worthington Biochemical, Freehold, NJ, 300 U/mg) in a water bath shaking at 37°C. Enzyme-digested cells were harvested after each 10-min digestion period. After 5 digestion periods, all the digested cells were then centrifuged at 1000 rpm for 10 min. Isolated fibroblasts were re-suspended in the cold (~4 °C) cardioplegic solution modified from a previous recipe [3] containing (in mM) 110 NaCl, 16 KCL, 16 $MgCl_2$, 2 mM Ca^{2+}, 10 HEPES, and 10 mM glucose, and immediately delivered to the Yue lab. Upon arrival to the Yue lab, fibroblasts were re-suspended in DMEM media, and seeded on the coverslips for patch-clamp experiments within 12~18 h, or seeded in the cultured dish and cultured for overnight for other experiments.

Culture of fibroblasts

Some fibroblasts isolated from CTL were seeded in the culture dish, and were cultured in the CO_2 cell culture incubator for the experiments of TGFβ1 treatment. Fibroblasts were cultured for overnight, and then serum starved for 12 h, followed by a 24-h incubation in the presence or absence of 10 ng/ml TGFβ1 in 1% serum DMEM medium [17]. After 24 h, fibroblasts were used for patch-clamp recording of TRPM4 currents.

Real-time RT-PCR

Real-time RT-PCR was conducted as we previously reported [17]. In brief, fibroblasts were collected and total RNA was extracted by TRIzol (Invitrogen). Real-time PCR was performed with the SYBR Green method following the protocol suggested by vendor (ABI). Human β-actin was used as an internal control. Primers used for real-time PCR are as follows: TRPM4: forward: TGCGCGCCGAGATGTAT, reverse: AAAGAAGCAGGTCGCTCCAG; β-actin: forward: CACCATTGGCAATGAGCGGTTC, reverse: AGGTCTTTGCGGATGTCCACGT.

Generation of shRNA and knockdown of hTRPM4

TRPM4 shRNA (TRPM4-shRNA) and scramble shRNA (SC-shRNA) were generated as we previously reported [17]. Briefly, hTRPM4-specific shRNA sequence (GCACGACGTTCATAGTTGA: NCBI reference sequence: NM_001321283.2) [36], and scramble shRNA sequence (TGTGCTCCGAACGTGTAGT) [17] were cloned into GFP lenti-virus vector (pLVTHM) using MluI and ClaI sites as we described in the previous study [17]. Lenti-viruses were generated in HEK-293 cells and were used to infect CTL human ventricular fibroblasts in culture. The effects of shRNA were evaluated by a patch clamp of GFP fluorescence fibroblasts 60 to 72 h after lenti-virus infection [17].

Electrophysiology

Whole-cell currents were recorded using an Axopatch 200B amplifier. A voltage ramp ranging from −120 mV to +100 mV at the interval of 1 to 5 s was used to elicit TRPM4 currents. Data were digitized at 5 or 10 kHz, and digitally filtered offline at 1 kHz. Patch electrodes were pulled from borosilicate glass and fire-polished to a resistance of ~3 MΩ when filled with internal solutions. Series resistance (Rs) was compensated up to 90% to reduce series resistance errors to <5 mV. Cells with Rs bigger than 10 MΩ were discarded. A fast perfusion system was used to exchange extracellular solutions, with complete solution exchange achieved in about 1 to 3 s. The internal pipette solution for TRPM4 whole-cell current recordings contained (in mM) 145 Cs-methanesulfonate ($CsSO_3CH_3$), 8 NaCl, 1 EGTA, and 10 HEPES, with pH adjusted to 7.2 with CsOH. Ca^{2+} was adjusted to various concentrations based on calculation using MaxChelator (http://www.stanford.edu/~cpatton/webmaxcS.htm). The standard extracellular Tyrode's solution for whole-cell recording contained the following (mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 Mg^{2+}, 10 HEPES, and 10 glucose. pH was adjusted to 7.4 with NaOH. The 145-mM NaCl was replaced by $NaSO_3CH_3$ in low Cl^- Tyrode solution for experiments to determine the potential contribution of the chloride channel to the current recordings, or by N-Methyl-D-glucamine (NMDG) chloride for leak detection. Isotonic Ca^{2+} solution contained 120 mM Ca^{2+}, 10 mM HEPES, and 10 mM glucose, with pH adjusted to pH 7.4 as we previously reported [39].

Immunoblotting

For western blot analysis, tissue lysates were separated on either 8% or 10% polyacrylamide gels as we reported previously [16]. This was followed by transferring to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST and

then incubated with anti-TRPM4 (Abcam, ab123936) or anti-GAPDH (Sigma, G-9545) antibodies. Blots then were washed with TBST and incubated with HRP-conjugated anti-rabbit or anti-mouse antibodies (Cell Signaling Tochnology, CS7076s and CS7074s). Blots then were washed, enhanced chemiluminescence (ECL; Pierce) was used for detection of signal, and images were captured by using a Fuji LAS-3000 Imaging System.

Data analysis

Pooled data are presented as mean±SEM. Concentration–response curves were fitted by an equation of the following form: $E = E_{max}\{1/[1 + (EC_{50}/C)^n]\}$, where E is the effect at concentration C, E_{max} is the maximal effect, EC_{50} is the concentration for the half-maximal effect, and n is the Hill coefficient [72]. EC_{50} is replaced by IC_{50} if the effect is an inhibitory effect. Statistical comparisons were analyzed using two-way analysis of variance (ANOVA) and a two-tailed t test with Bonferroni correction; $p < 0.05$ indicated statistical significance.

Results

Functional expression of TRPM4 in human ventricular fibroblasts

Several TRP channels have been shown to express in cardiac fibroblasts [22, 33]. In order to investigate whether TRPM4 is functionally expressed in human cardiac fibroblasts, we applied whole-cell current recording using pipette solutions containing 100 μM Ca^{2+} to activate TRPM4, and 3 mM Mg^{2+} to eliminate potential contamination from the TRPM7 current. As shown in Fig. 1, TRPM4 was activated with time while pipette Ca^{2+} was dialyzed into the cell (Fig. 1a). NMDG was used to make sure that there was no leak current, and a low-chloride Tyrode solution was used to ensure no Cl^- contamination in the current recordings. To determine whether the recorded current was TRPM4 current, we applied extracellular isotonic Ca^{2+} solution to examine ionic permeation. The isotonic calcium solution did not produce any inward current, indicating that the current recorded in the Tyrode solution was carried by monovalent cations. Among the two calcium nonpermeable TRP channels, TRPM4, but not TRPM5, is highly expressed in the heart [23, 69], suggesting that the currents we recorded were likely carried by TRPM4. We further confirmed that it was TRPM4 current by applying 9-phenanthrol (9-phen) (Fig. 1a, b), which inhibits TRPM4 but not TRPM5 [25]. To further confirm that the recorded currents were TRPM4 currents, we applied TRPM4-shRNA to knock down TRPM4. As shown in Fig. 1c, d, TRPM4-shRNA produced about 80% inhibition, indicating that the recorded current which was blocked by 9-phen was indeed TRPM4 currents.

TRPM4 current is upregulated in heart failure patients

TRPM4 has been shown to play a role in cardiac myocytes under pathological conditions. In order to investigate whether TRPM4 in fibroblasts plays a role under pathological conditions, we determined to investigate if TRPM4 in fibroblasts is regulated by heart failing pathological conditions. Human left ventricular fibroblasts from heart failure patients (HF) and control patients (CTL) were isolated. Using the TRPM4 recording conditions shown in Fig. 1, we did current recording in fibroblasts freshly isolated from left ventricles of CTL and HF patients. The TRPM4 currents recorded from CTL and HF fibroblasts displayed a similar activation process and linear I^- V relation after activation (Fig. 2a-d). However, the

current amplitude in fibroblasts from HF patients was significantly bigger than that from CTL fibroblasts (Fig. 2e, f).

Calcium sensitivity of TRPM4 in CTL and HF fibroblasts

In order to understand the mechanisms underlying the enhanced current amplitude in the HF fibroblasts, we investigated calcium sensitivity for TRPM4 activation. Intracellular pipette solutions with various Ca^{2+} concentrations were used to activate TRPM4 currents in fibroblasts from both CTL and HF patients (Fig. 3a and c). Concentration-dependent analysis yielded EC_{50} of 15.5±0.6 μM and 14.4±0.8 μM for activation of TRPM4 in CTL and HF fibroblasts, respectively, indicating that the calcium sensitivity of TRPM4 was not changed by heart failure conditions.

Effects of 9-phen on TRPM4 from fibroblasts

To further characterize the properties of TRPM4 in fibroblasts from CTL and HF left ventricular fibroblasts, we compared inhibitory effects of 9-phen on TRPM4. TRPM4 currents were recorded with 100 μM Ca^{2+} in the pipette solution (Fig. 4). We found that 9-phen inhibited TRPM4 in a concentration-dependent manner, with IC_{50} of 20.7±2.9 μM and 19.5±5.7 μM, respectively, in CTL and HF left ventricular fibroblasts. Thus, TRPM4 channels from CTL and HF left ventricular fibroblasts have similar sensitivity to 9-phen inhibition.

Upregulation of TRPM4 expression in heart failure patients

Since the increased currents of TRPM4 are independent on intracellular calcium sensitivity, we investigated the expression level of TRPM4 in CTL and HF patients. As the limited number of fresh isolated or cultured fibroblasts does not provide enough proteins, we used left ventricular tissue from CTL and HF patients for western blot experiments. The frozen left ventricular tissues from the same HF patients or CTL as the current recording were used for protein extraction. As shown in Fig. 5, the protein level of TRPM4 in the HF patients was significantly higher than that of CTL (Fig. 5a, b), consistent with the increased functional change of TRPM4 reflected by current amplitude. Moreover, using RNA extracted from the same batches of CTL and HF fibroblasts used for patch-clamp experiments, we did a relative quantification of mRNA expression levels by qPCR. As shown in Fig. 5c, the mRNA level of TRPM4 in HF fibroblasts is more than three-fold higher than that of CTL fibroblasts.

Regulation of TRPM4 by TGFβ1

The enhanced TRPM4 currents as well as mRNA and protein expression levels in the HF patients suggest that TRPM4 is upregulated by pathological conditions in vivo in heart failure patients. Among various pathogenesis factors associated with heart failure, cardiac fibrosis plays a detrimental role in heart failure. We therefore investigated whether fibrosis-promoting factor TGFβ1 plays a role in TRPM4 upregulation. Fibroblasts isolated from the CTL left ventricle were cultured and, after serum starving, were treated with 10 ng/ml TGFβ1. Current was recorded 24 h after TGFβ1 treatment. Current density of TRPM4 was increased from 22.7 pA/pF in nontreated fibroblasts to 42.3 pA/pF after TGFβ1 treatment

(Fig. 6a, b), indicating that TRPM4 is upregulated by fibrotic conditions. This result is consistent with the upregulated mRNA level of TRPM4 in mouse fibroblasts after treatment with TGFβ1 [17]. Whereas TGFβ1 treatment for 24 h upregulated TRPM4 expression, acute application of TGFβ1 to CTL fibroblasts during patch-clamp experiments did not change the current amplitude (Fig. 6c, d). Thus, it is conceivable that TRPM4 expression is upregulated during the pathogenesis process of heart failure, and the upregulated TRPM4 may have in turn contributed to fibrogenesis in the progression of heart failure.

Discussion

In this study, we demonstrate an increased current amplitude of TRPM4 in left ventricular fibroblasts from heart failure patients in comparison with CTL individuals. The increased TRPM4 current is not due to the changes of intracellular calcium sensitivity of TRPM4 but is the result of upregulation of the TRPM4 channel protein in HF patients versus CTL individuals. Other properties of TRPM4 channels, such as I–V relation and the sensitivity to extracellular 9-Phen blockade, display no differences between TRPM4 channels recorded in fibroblasts from HF patients and CTL individuals. Moreover, TRPM4 expression in fibroblasts from CTL can be potentiated by TGFβ1, suggesting that TRPM4 may be regulated by fibrogenesis conditions in vivo. Our results of upregulated functional TRPM4 channels in human HF fibroblasts suggest that TRPM4 may play a role in influencing cardiac fibrogenesis cascade, thereby contributing to the pathogenesis of HF.

TRPM4 is a wildly expressed Ca^{2+}-impermeable monovalent cation channel [9, 50, 69]. Long before the cloning of TRPM4 gene, a Ca^{2+}-activated nonselective cation current was recorded in various types of cells including cardiac cells [10, 21, 54]. To date, the TRPM4-like Ca^{2+}-activated nonselective endogenous cation currents have been well characterized in sino-atrial node cells [13], cardiac atrial myocytes [28], Purkinje fiber [34], and ventricular myocytes [29]. The functional TRPM4 expression in the conduction system and cardiac myocytes strongly support the role of TRPM4 in cardiac electrical activity and in the pathogenesis process of hypertrophy and heart failure [27, 41, 73].

In comparison with the functional expression and the known pathophysiological functions of TRPM4 in excitable cells in the heart, little is known about TRPM4 in the cardiac fibroblasts. In cardiac fibroblasts, although mRNA expression of TRPM4 has been detected in human and mouse atrial fibroblasts [17], functional TRPM4 expression in human atrial fibroblasts was not reported until very recently by Simard and colleagues [64]. In the present study, using human ventricular fibroblasts, we demonstrate that TRPM4 is also functionally expressed in human ventricular fibroblasts. We show that the whole cell currents of TRPM4 in CTL and HF exhibit a concentration-dependent activation by intracellular Ca^{2+} and concentration-dependent block by extracellular application of 9-Phen. More importantly, we found that TRPM4 current density in HF fibroblasts is more than 2-fold bigger than that of CTL fibroblasts.

Our result showing the functional expression of TRPM4 in human ventricular fibroblasts and upregulation by heart failure is the first report to suggest that TRPM4 in cardiac fibroblasts may play a role in the pathogenesis of human heart failure. Consistent with our results of

upregulation of TRPM4 in HF fibroblasts, a recent study which thoroughly analyzed the TRP channel in the failing hearts demonstrated that the TRPM4 mRNA level in the left ventricles of a cohort containing 43 patients was 65% higher than that of nonfailing control patients [15]. Given the important role of fibrosis in causing various types of heart diseases, it is plausible that TRPM4 may play a role in fibroblast proliferation and differentiation, thereby contributing to cardiac fibrogenesis. Indeed, in human atrial fibroblasts, Simard and colleagues nicely showed that TRPM4 influences human atrial fibroblast growth [64]. In the present study, we found that TGFβ1, a strong fibrosis-promoting cytokine, increases the TRPM4 current density after a 24-h treatment. In agreement with this result, we previously demonstrated that TGFβ1 treatment enhanced the TRPM4 mRNA level in mouse fibroblasts [17]. The effects of TGFβ1 on increasing TRPM4 expression in the cultured fibroblasts may represent a recapitulation of the in vivo microenvironment where fibroblasts in human ventricles are stimulated by cytokines such as TGFβ1 during the development and progression processes of hypertrophy and heart failure.

TRPM4 is a monovalent cation channel. Unlike the Ca^{2+}-permeable TRP channels [37, 74], such as TRPC3 [31, 55], TRPV4 [59], and TRPM7 [17], which have been shown to play a role in fibrogenesis cascade, it is surprising that the Ca^{2+}-impermeable TRPM4 is highly expressed in cardiac fibroblasts and is drastically upregulated in heart failure patients. How could TRPM4 influence fibroblast biological function and be involved in the fibrogenesis process? TRPM4 conducts inward Na^+ current at negative membrane potentials and outward K^+ currents at positive membrane potentials under physiological ionic conditions; therefore, TRPM4 can contribute to depolarize fibroblasts as well as hyperpolarize fibroblasts. By depolarizing or hyperpolarizing fibroblasts, TRPM4 can influence Ca^{2+} signaling mediated by Ca^{2+}-permeable TRP channels, such as TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6, as well as TRPM2 and TRPM7, in human ventricular fibroblasts [15]. Indeed, TRPM4 has been shown to regulate calcium oscillation in different cell types such as T-cells, β-pancreatic cells, and HL-1 cells [5, 43]. Thus, TRPM4 may indirectly influence Ca^{2+} homeostasis in HF fibroblasts. Alternatively, as the voltage-gated Na^+ channel (NaV1.5) only expresses in differentiated myofibroblasts [6], TRPM4 may serve as a depolarization channel in cardiac fibroblasts and therefore contribute to biological function of fibroblasts under normal physiological conditions. Nonetheless, detailed mechanisms by which TRPM4 regulates fibroblast function and fibrosis process need further investigation.

In the present study, we used freshly isolated fibroblasts to evaluate the functional TRPM4 expression level in order to closely reflect the TRPM4 expression level *in vivo*. However, due to the limited number of isolated fibroblasts, for some experiments, such as western blot, we used left ventricular tissues in order to get enough protein. Moreover, we have only six samples from HF patients and CTL individuals, which did not allow us to investigate whether there is a difference in TRPM4 expression between male and female, as well as whether there is age-dependent difference in TRPM4. Although there are limitations in our study, the discovery in our present study provide novel information about the potential role of TRPM4 in human fibroblasts as well as in human heart failure, and suggest that TRPM4 may serve as a novel target for treating heart failure in patients with cardiac conduction problem.

In summary, our data represent the first report demonstrating the functional expression of TRPM4 in human left ventricular fibroblasts, and the enhanced TRPM4 channel function in the heart failure patients versus control individuals without heart failure. Given the important role of TRPM4 in the cardiac conduction system, our data provide novel insights that targeting on TRPM4 may provide better therapy for heart failure patients complicated with conduction problems.

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Abbreviations

BMI	body mass index
CAD	coronary artery disease
DM	diabetes mellitus
EF	ejection fraction
Eth	ethnicity
Gen	gender
HF	heart failure
HLD	hyperlipidemia
HTN	hypertension
Med	out-patient medication
MI	myocardial infarction
VD	valve diseases

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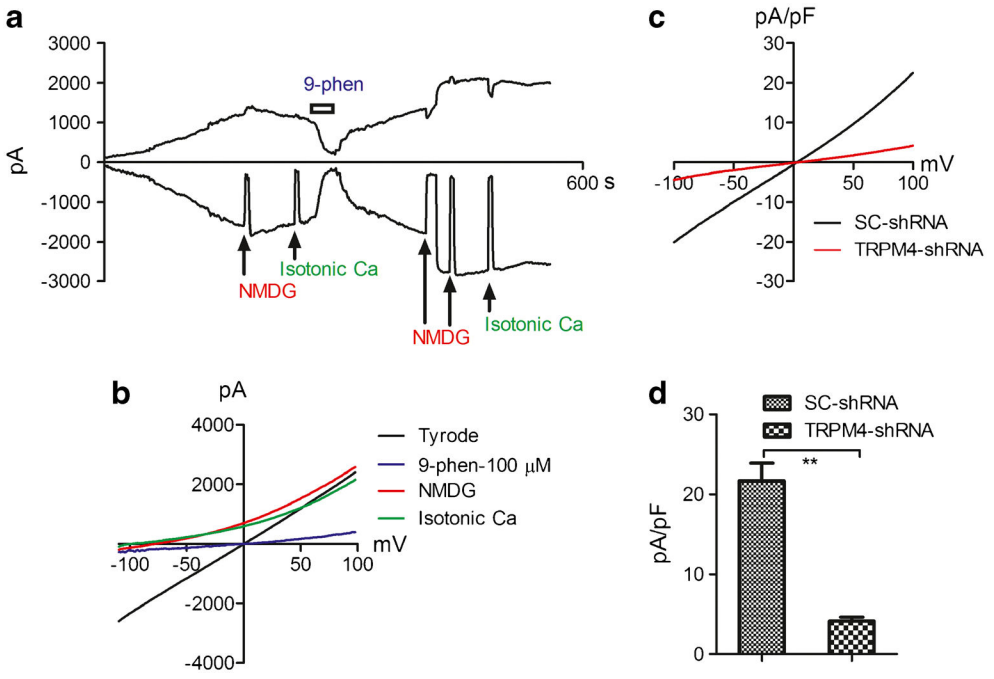

Fig. 1.

Functional expression of TRPM4 in human ventricular fibroblasts. **a** Time-dependent activation of TRPM4 recorded in a HF fibroblast, and inhibition by 100 μM 9-phen. Inward and outward currents were measured at −100 mV and +100 mV. TRPM4 currents were elicited by a ramp protocol ranging from −120 to +100 mV with the recording condition of 100 μM Ca^{2+} in the pipette solution. NMDG was applied to make sure that there was no leak current throughout the recording time. Isotonic Ca^{2+} solution was used to detect whether there was any Ca^{2+} permeation. **b** Representative recordings of TRPM4 in Tyrode solution and after blockade by 9-phen. Note that inward current was eliminated by NMDG and isotonic Ca^{2+} solution, indicating that there was no leak current and no Ca^{2+} permeability. **c** Representative TRPM4 currents recorded in CTL fibroblasts treated with TRPM4-shRNA or SC-shRNA. **d** Average current amplitude of TRPM4 in fibroblasts treated with TRPM4-shRNA (4.1±0.4 pA/pF, n=11) or SC-shRNA (21.7±2.2, n=12). Note that TRPM4 currents were largely eliminated by TRPM4-shRNA (**p<0.01)

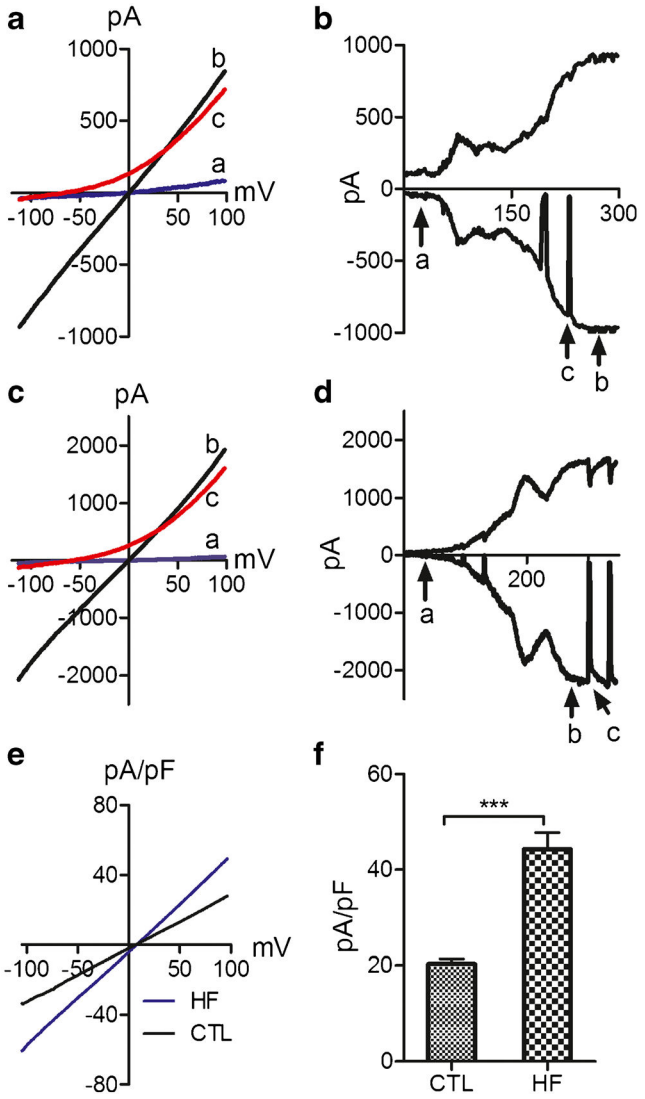

Fig. 2.

Comparison of TRPM4 currents recorded from fibroblasts of control individuals (CTL) and heart failure patients (HF). **a** Representative recordings of TRPM4 before and after activation, as well as after NMDG application at the corresponding time points (**a, b, c**) as shown in **b** from CTL fibroblasts. **b** Time-dependent activation of TRPM4 from CTL fibroblasts. Inward and outward current were measured at −100 and +100 mV respectively. **c** Representative traces of TRPM4 before and after activation, as well as after NMDG application at the corresponding time points (**a, b, c**) as shown in **d** from an HF fibroblast. **d** Time-dependent activation of TRPM4 from HF fibroblasts. Inward and outward currents were measured at −100 and +100 mV, respectively. **e, f** Comparison of TRPM4 current density from representative cells (**c**), and average current density of CTL (n=50) and HF (n=38) fibroblasts isolated from 6 CTL and HF hearts. Current density from HF fibroblasts is significantly bigger than that of CTL fibroblasts (***p<0.001)

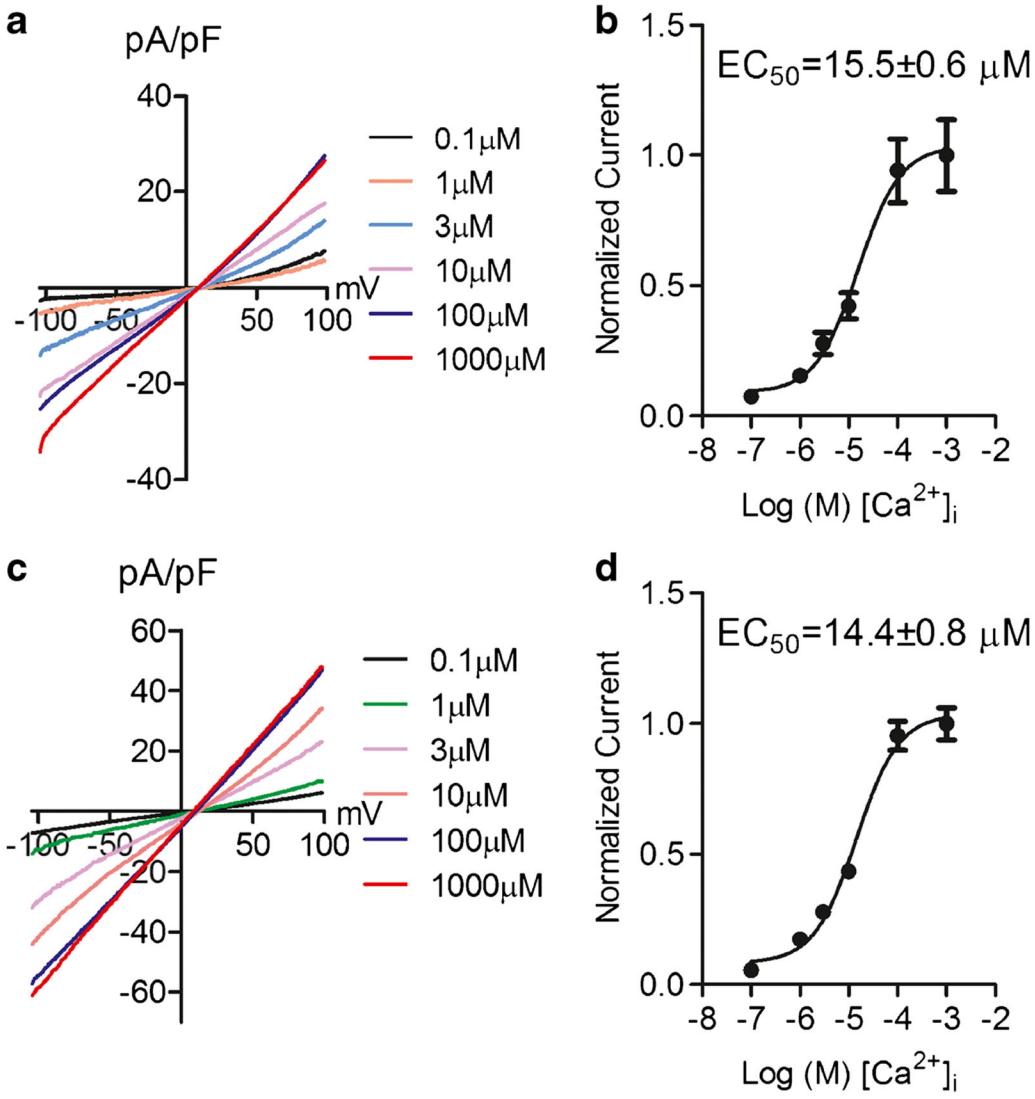

Fig. 3.

Ca^{2+}-sensitivity of TRPM4 from CTL and HF fibroblasts. **a, c** Representative TRPM4 currents recorded with various concentrations of intracellular Ca^{2+} in CTL (**a**) and HF (**c**) fibroblasts. **b, d** Concentration-dependent effects of intracellular Ca^{2+} on TRPM4. Dose–response curves yielded EC_{50} of 15.5±0.6 μM for CTL fibroblasts (**b**) and 14.4±0.8 μM for HF fibroblasts (**d**), respectively. No significant difference in EC_{50} was observed (n=6 for each Ca^{2+} concentration in CTL and HF groups)

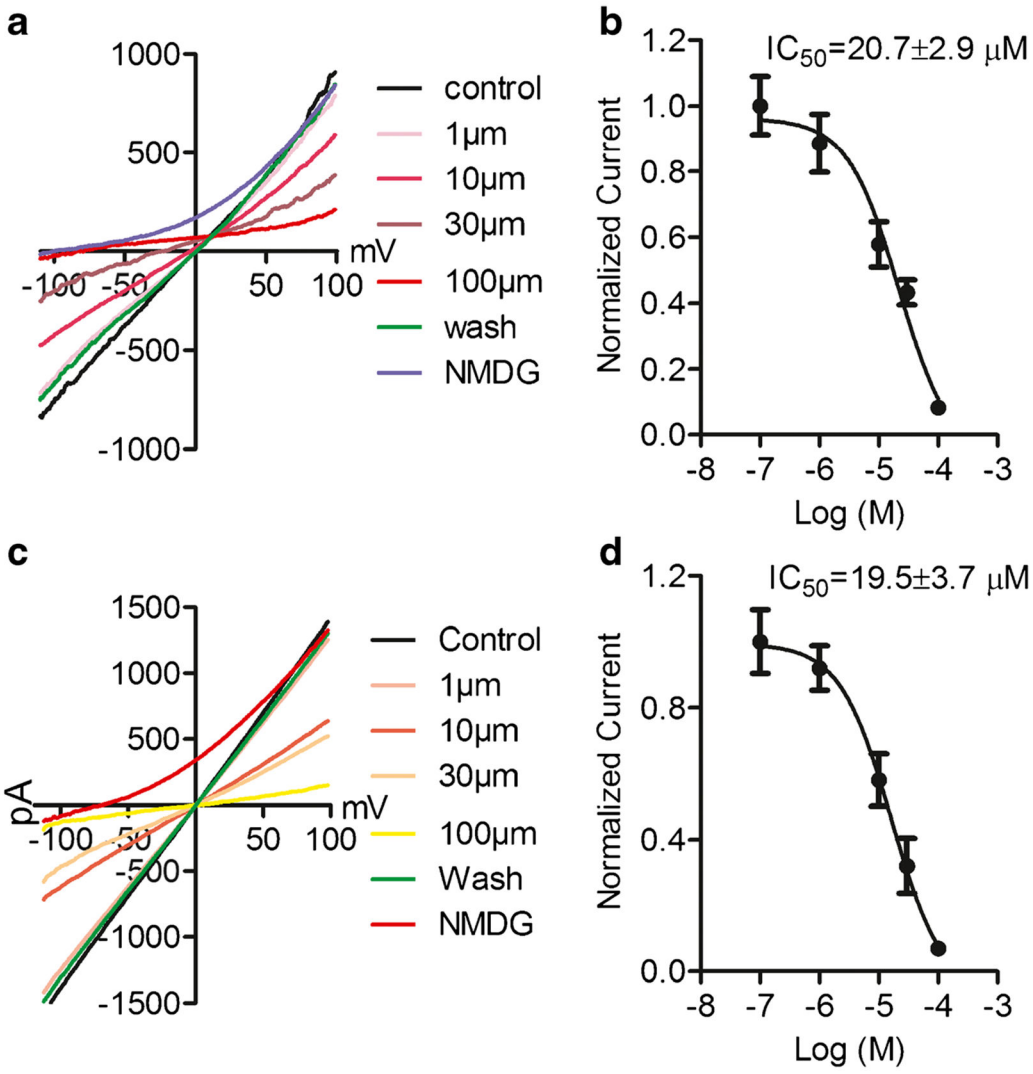

Fig. 4.

Concentration-dependent effects of 9-phen on TRPM4 currents recorded from CTL and HF fibroblasts. **a, c** Representative recordings of TRPM4 current elicited by voltage ramp ranging from −120 to +100 mV. Effects of 9-phen at 0.1, 1, 10, 30, and 100 μM were tested in CTL (**a**) and HF (**c**) fibroblasts. The effect of 9-phen was reversal. **b, d** Dose–response curves constructed from normalized current amplitude yielded IC_{50} of 20.7±2.9 μM for CTL fibroblasts (**b**) and 19.5 ±3.7 μM for HF fibroblasts (**d**), respectively. No significant difference in IC_{50} was observed (n=6 for each group)

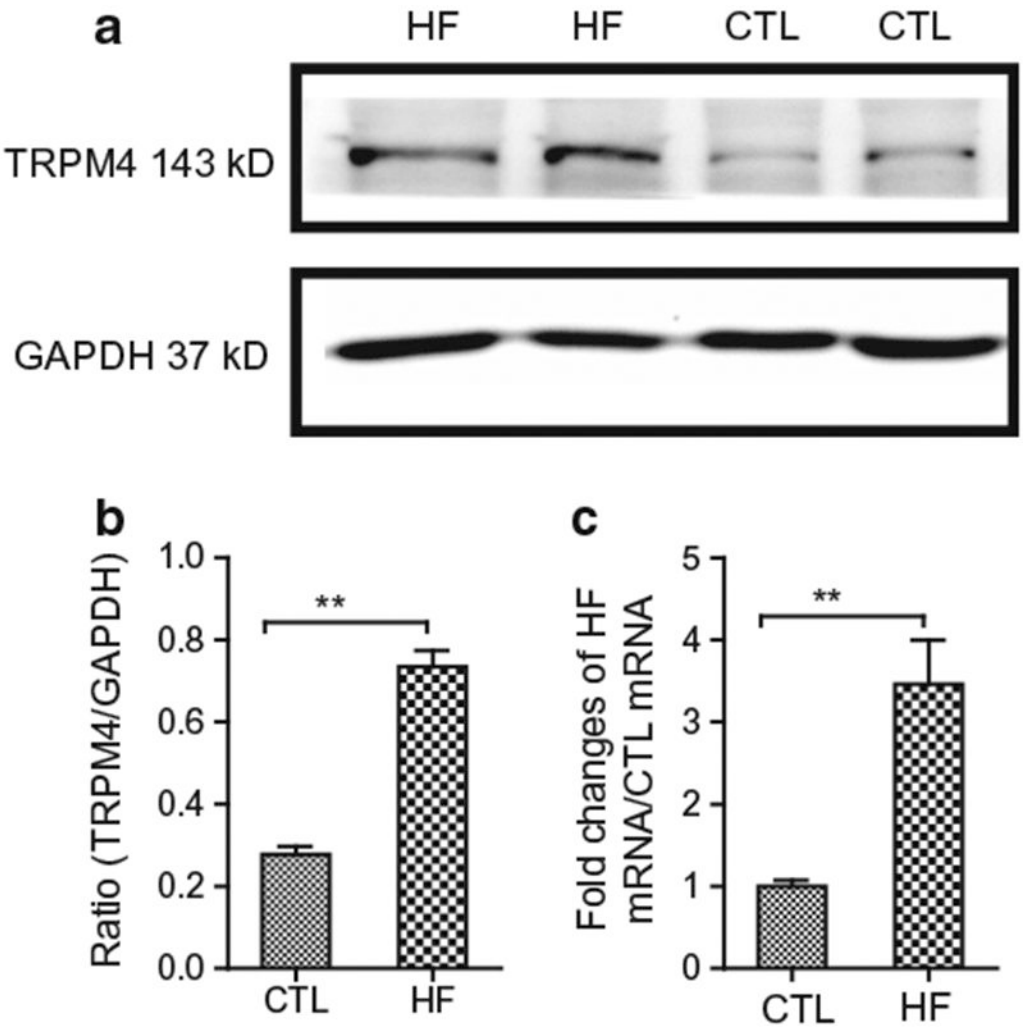

Fig. 5.

TRPM4 expression detected by western blot (WB) in CTL and HF left ventricle. **a** WB results of TRPM4 and loading control using GAPDH. **b** Average ratio of TRPM4 versus GAPDH in CTL and HF hearts. Expression of TRPM4 was significantly bigger in HF hearts than that in CTL hearts (**p<0.01, n=6 for each group). **c** qPCR quantification of TRPM4 expression levels in fibroblasts of CTL and HF. RNA samples were extracted from the same batches of fibroblasts used for patch-clamp experiments (**p<0.01, n=6 for each group)

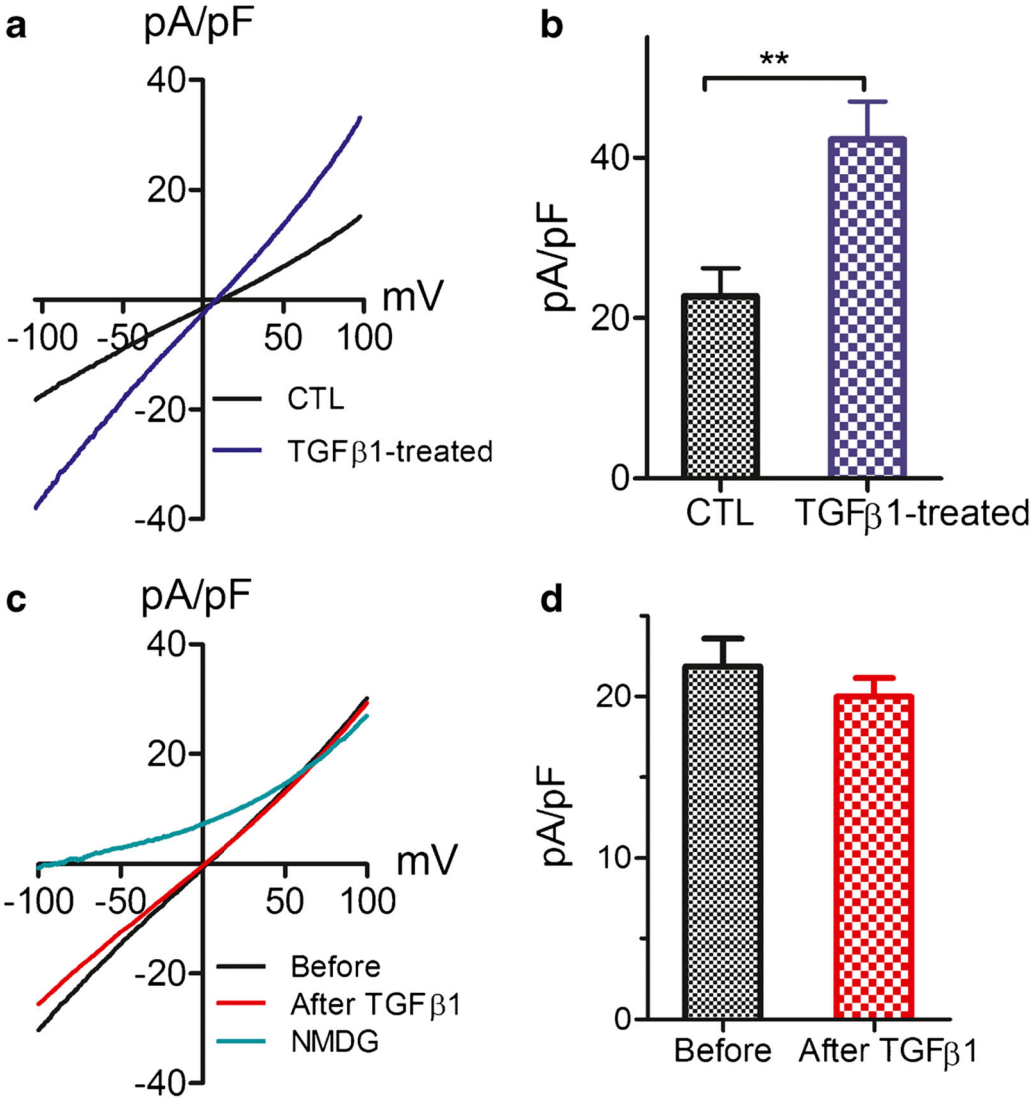

Fig. 6.

Effect of TGFβ1 on TRPM4 in CTL fibroblasts. **a** Representative TRPM4 recorded from CTL fibroblasts 24 h after treatment with 10 ng/ml TGFβ1 (blue trace) in comparison with current recorded from nontreated control fibroblasts. **b** Average current density of TRPM4 in TGFβ1-treated (n=16) and nontreated (n=14) fibroblasts (**p<0.01). **c** TRPM4 currents recorded from CTL fibroblasts before and after perfusion with 10 ng/ml TGFβ1. NMDG was used to monitor leak current. **d** Mean current density of TRPM4 before and after TGFβ1 perfusion. No statistical difference was observed (n=8)

Table 1

Patients' clinical characteristics

	Non-HF (CTL)	HF
N	6	6
Age (years)	52.8±3.3	42.8 ±4.9
Male (%)	33.3	83.3
Cau (%)	50	50
AA (%)	33.3	16.7
Hisp (%)	16.7	0
Unknown race (%)	0	33.3
BMI	31.9 ± 2.8	33.1± 3.4
EF (%)	62.2 ± 3.6	14.5±2.5
HF (%)	0	100
CAD (%)	0	50
MI (%)	0	0
VD (%)	0	0
HTN (%)	66.7	50
HLD (%)	33.3	50
DM (%)	16.7	16.7