

TRPC1 expression and distribution in rat hearts

H. Huang, W. Wang, P. Liu, Y. Jiang, Y. Zhao, H. Wei, W. Niu

¹Department of Physiology, Capital Medical University, Beijing, China



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Transient receptor potential canonical (TRPC) proteins have been identified as a family of plasma membrane calcium-permeable channels. TRPC proteins can be activated by various stimuli and act as cellular sensors in mammals. Stretch-activated ion channels (SACs) have been proposed to underlie cardiac mechano-electric feedback (MEF), although the molecular entity of SAC remains unknown. There is evidence suggesting that transient receptor potential canonical 1 (TRPC1) is a stretch-activated ion channel. As a non-selective cation channel, TRPC1 may cause stretch-induced depolarization and arrhythmia and thus may contribute to the MEF of the heart. In this study, we examined the expression patterns of TRPC1 in detail at both the mRNA and protein levels in rat hearts. We isolated total RNA from the left and right atria, and the left and right ventricles, and detected TRPC1 mRNA in these tissues using reverse-transcriptase polymerase chain reaction (RT-PCR). To study the protein localization and targeting, we performed immunohistochemistry and immunofluorescence labeling with the antibody against TRPC1. TRPC1 was detected in the cardiomyocytes of the ventricle and atrium at both the mRNA and protein levels. The cell membrane and T-tubule showed strong fluorescence labeling in the ventricular myocytes. Purkinje cells, the endothelial cells and smooth muscle cells of the coronary arterioles also displayed TRPC1 labeling. No TRPC1 was detected in fibroblasts. In conclusion, TRPC1 is widely expressed in the rat heart, including in working cells, Purkinje cells and vascular cells, suggesting that it plays an important role in the heart. The specific distribution pattern offered a useful insight into its function in adult rat ventricular cells. Further investigations are needed to clarify the role of TRPC1 in regulating cardiac activity, including cardiac MEF.

Key words: TRPC1, Heart, Expression and distribution.

Correspondence: Weizhen Niu,
Department of physiology, Capital Medical University,
You An Men Wai Street Xitoutiao 10, Beijing 100069, China
Tel: +86.10.83911470.
E-mail: niuwz@yahoo.cn

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The heart not only functions as a pump but also senses the variational stress on itself during the cardiac cycle. Proper mechanical loading is necessary for the development and maturation of the heart and to maintain normal function (Tobita and Keller, 2000). However, overloading or mechanical stimulus causes cardiac hypertrophy and arrhythmias (Clemo *et al.*, 1998; Sadoshima *et al.*, 1992a; Schrickel *et al.*, 2002). Numerous studies have shown that a mechanical stretch or load applied to a cardiac tissue can induce significant electrophysiological changes via the process termed "mechano-electric feedback" (MEF). The underlying mechanisms linking such a mechanical impact to subsequent arrhythmias remain unknown.

Mechanosensitive channels are proving germane to our understanding of cardiac MEF. The electrophysiological changes during MEF have been principally attributed to the activity of stretch-activated ion channels (SACs) (Hu and Sachs, 1997), whose open probability was augmented with increasing membrane tension. Two groups of SACs in the heart have been reported: stretch-activated potassium channels (SAKCs) and stretch-activated non-selective cation channels (SACCs). Recent studies suggest that the two-pore domain potassium channel TREK-1 may be the molecular entity of SAKCs in the heart (Liu *et al.*, 2008; Tan *et al.*, 2004; Xian Tao *et al.*, 2006). However, the identities of the SACCs remain unknown. SACCs transduce membrane stretch into cation (Na^+ , K^+ , Ca^{2+} and Mg^{2+}) fluxes across the cell membrane and are implicated in both stretch-induced depolarization and cardiac arrhythmias (Franz *et al.*, 1992; Wei *et al.*, 2006). Identifying the molecular components of SACCs and elucidating the mechanisms of activation are of equal importance, and both tasks have proven difficult.

In recent years, attention has been focused on the idea that the transient receptor potential (TRP) channels act as cellular sensors (Clapham,

2003). Particularly, the TRP-related family members OSM-9, OTRPC4 and NOMPC have been implicated in osmoreception (Clapham, 2003). As non-selective cation channels, TRP channels with mechanosensitivities may play an important role in MEF. Canonical TRP (TRPC) channels are a group of mammalian Ca^{2+} -permeable channels that share homology with the *Drosophila* TRP channels (Minke and Cook, 2002). TRPC channels mediate store-operated Ca^{2+} entry as well as store-independent Ca^{2+} influx (Kwan et al., 2004; Minke and Cook, 2002; Nilius *et al.*, 2003). TRPC channels are unlikely to play an important role in relation to the $[\text{Ca}^{2+}]_i$ fluctuations responsible for excitation-contraction coupling. Rather, they are more likely to control local $[\text{Ca}^{2+}]_i$, which may play a role in regulating the signaling pathways that contribute to positive inotropic responses, arrhythmias, cardiac damage following ischemia-reperfusion, myocyte hypertrophy and changes in gene expression (Ohba *et al.*, 2007).

Aside from the well-known voltage-gated calcium channels (VGCCs), TRP proteins have attracted almost exponentially growing attention as exceptionally unique non-voltage-gated Ca^{2+} -entry channels activated by a multitude of physiochemical stimuli and associated with a diversity of biological functions. Most recently, a member of the TRPC subfamily, TRPC1, was confirmed to be mechanosensitive and to form the SACC in vertebrate cells (Maroto *et al.*, 2005). Although another study showed that overexpression of hTRPC1 in mammalian cells does not increase the SACC activities significantly, one of the reasons may be that plasma membrane TRPC1 does not increase in this study (Gottlieb *et al.*, 2008). The factors involved in regulating TRPC1 trafficking as well as TRPC1 channel function are complicated and worthy of further study.

Anyway, TRPC1 is one of the most studied TRP channels (Wes *et al.*, 1995; Zhu *et al.*, 1995; Zitt *et al.*, 1996). At the cellular level, TRPC1 functions as a pathway of non-voltage-gated Ca^{2+} and Na^+ influxes that may also include the stretch-activated cation influx. Thus, clarifying the expression and distribution of TRPC1 in the heart is a necessary step to offer insight into whether or not TRPC1 can act as a SACC in the heart.

Studies directly and detailedly demonstrating TRPC1 localization and targeting in intact rat hearts are lacking except a few reverse-transcrip-

tion polymerase chain reaction (RT-PCR) experiments. In the present study, we have combined RT-PCR and immunohistochemistry to demonstrate the overall expression patterns of TRPC1 in the rat heart. Our data provide the reference information both for further identification of TRPC1 as potential candidate SACC in the rat heart and for further investigation of TRPC1 related cardiac MEF.

Materials and Methods

RT-PCR

Total RNA was extracted from left ventricle, right ventricle, left atrium and right atrium of adult Sprague-Dawley (SD) rats (230-250 g) respectively, using the trizol-chloroform-isopropyl alcohol method (Invitrogen, Carlsbad, USA). RT-PCR was performed using a two-step RT-PCR kit (Takara RNA PCR Kit (AMW) Ver. 3.0, Takara, Otsu, Japan). Total RNA was reversely transcribed into first-strand cDNA using oligo-dT primers and AMV reverse transcriptase (Takara, Otsu, Japan). Reverse transcription was performed at 42°C for 30 minutes, followed by a final terminal reaction at 99°C for 15 minutes. The cDNA products were used as templates for PCR amplification, which was performed with Taq DNA polymerase (Takara, Otsu, Japan). The primers for PCR were designed according to the sequence of rat TRPC1 mRNA available in the GenBank database (access number: NM_053558). The primer pair (forward/reverse) was: 5'-CTC TTG ACA AAC GAG GAC TAC TA-3' (in exon 5)/ 5'-GTC TTC CAA CCC TTC ATA CCA-3' (in exon 7). Cycling conditions were as follows: 2 minutes at 94°C followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C and a final extension of 7 minutes at 72°C. Control reactions without template RNA or the reverse transcriptase were included for each PCR amplification experiment. PCR products were separated on 1.5% agarose gels by electrophoresis and visualized by staining with ethidium bromide. The authenticity of amplified PCR products was verified using an ABI PRISM DNA sequencing system (Perkin Elmer).

Immunohistochemistry

The heart of SD rat was used for immunohistochemical experiments. Immunoreactivity was tested using avidin-biotin-peroxidase reactions. Tissue

cross-sections of 3 μm were rehydrated in a graded alcohol series to 70% ethanol, washed with deionized water and then preincubated with 3% (v/v) H_2O_2 in absolute methanol in order to inhibit endogenous peroxidase activity. Normal goat serum was then used to block the endogenous biotin. Sections were incubated at 4°C overnight with rabbit anti-rat TRPC1 primary antibodies (1:100 dilution, batch number AN-04, Alomone Labs, Jerusalem, Israel). Secondary biotinylated goat anti-rabbit IgG was subsequently applied, the immunoreactivity was visualized with streptavidin-biotin-peroxidase using 3, 3'-diaminobenzidine (Sigma-Aldrich, St. Louis, USA) as a substrate, and the sections were counterstained with hematoxylin to show nuclei. In negative control experiments, the primary antibodies were either omitted or were preabsorbed for 2.5 hours at room temperature with a 10-fold molar excess of peptide antigens provided by the manufacturer. A positive control was performed on skeletal muscle as the positive tissue because the presence of TRPC1 in skeletal muscle had previously been confirmed (Vandebrouck *et al.*, 2002).

Immunofluorescence

Ventricular myocytes were enzymatically isolated from adult SD rat heart, as described previously (Niu and Sachs, 2003). Cells in suspension were transferred to slides, fixed in cold 4% paraformaldehyde solution for 15 minutes, permeabilized with 0.3% Triton X-100 for 10 minutes at room temperature, and preincubated with 3% (v/v) H_2O_2 in absolute methanol for 5 minutes. Normal goat serum was used to block endogenous biotin. Then the cells were exposed to primary (rabbit anti-rat TRPC1, 1:100 dilution, batch number AN-04, Alomone Labs, Jerusalem, Israel) and secondary (tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG, Jackson Labs, West Grove, USA) antibodies. Actin filaments were stained with 5 $\mu\text{g}/\text{mL}$ of Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, USA) at 4°C for 30 minutes. The myocytes were visualized using a confocal microsystem (LAS AF-TCS SP5, Leica, Wetzlar, Germany). Rhodamine (TRITC) was excited at 561 nm and detected at 585–640 nm. Alexa Fluor 488 phalloidin was excited at 495 nm and detected at 519 nm.

Results

RT-PCR-based detection of TRPC1 expression in rat hearts

RT-PCR was used to examine the expression of TRPC1 transcripts. Primers were designed according to the corresponding rat TRPC1 mRNA sequences (NM_053558). Forward and reverse primers for TRPC1 were located in separate exons. RT-PCR amplified the expected 467 base pair (bp) product indicative of TRPC1 from total RNA isolated from left ventricle, right ventricle, left atrium and right atrium of rat (Figure 1). The 467 bp product for TRPC1 did not result from genomic DNA contamination since PCR amplification from genomic DNA should result in products with a much larger molecular size. The product was absent in the control experiment, which was performed without template RNA or reverse transcriptase (data not shown). The authenticity of the 467 bp product was confirmed by DNA sequencing (*data not shown*).

Detection of TRPC1 in rat hearts by immunohistochemistry

Immunohistochemistry was used to explore the cellular localization of TRPC1 in the rat heart. Strong positive signals, brown in color, can be observed in the cardiomyocytes of ventricles (Figure 2A) and atria (Figure 2B), particularly on the cell membrane of the ventricular myocytes. The immunohistochemical studies also confirmed positive signals in the endothelial cells and the smooth muscle layers of coronary arterioles, although the staining was much weaker than that seen in cardiomyocytes (Figure 2C). Purkinje cells beneath the endocardium were also positively stained. Purkinje cells were characterized by their special shape and pigmentation via hematoxylin

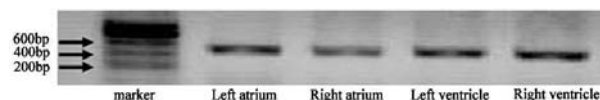


Figure 1 RT-PCR based detection of TRPC1 in rat hearts. PCR products were observed in ethidium bromide-stained agarose gel. TRPC1 DNA fragments (467 bp) were amplified from left atrium, right atrium, left ventricle and right ventricle of rats.

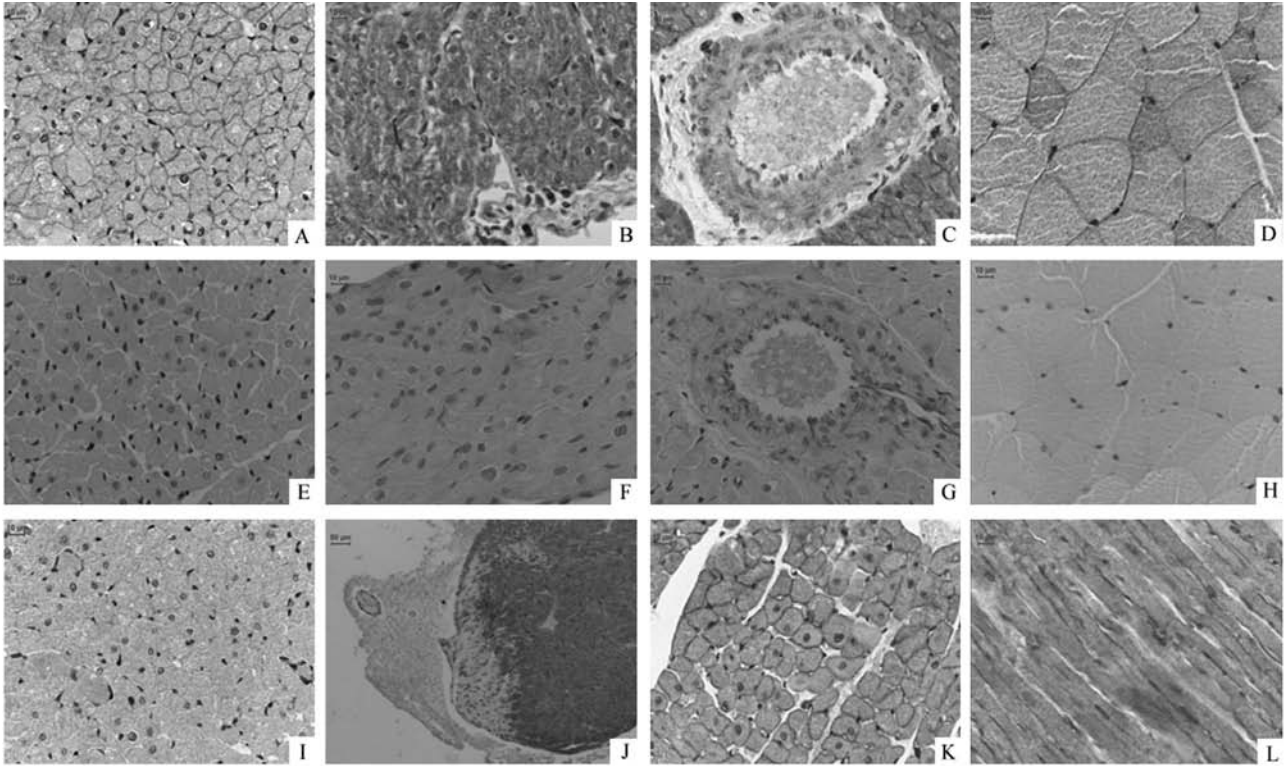


Figure 2. Immunohistochemical detection of TRPC1 protein in rat hearts. Sections were incubated with primary antibody for TRPC1 (A, B, C, D), without primary antibody (E, F, G, H) or with primary antibody preabsorbed by TRPC1 peptide for negative control (I). Positive signals in brown color can be visualized in the myocytes of the left ventricle (A) and atrium (B), endothelial and smooth muscle layers of coronary arterioles (C), and skeletal muscle cells (D), as positive control. No positive signal could be observed in control experiments without primary antibody. A faint signal was occasionally observed in antigen preabsorption control (I). There are negative cells in the edge of ventricular tissues (J) and also the fibroblasts between ventricular myocytes which showed blue nuclei without positive signals. The right ventricle shows the same distribution of TRPC1 positive signal (K) as the left ventricle. TRPC1 showed intense staining on the cell membranes of ventricular myocytes (A, K, L) and skeletal muscle cells (D). The longitudinal section of left ventricle also shows striated distribution of TRPC1 (L). Scale bar =10 μm, except scale bar = 50 μm in panel J.

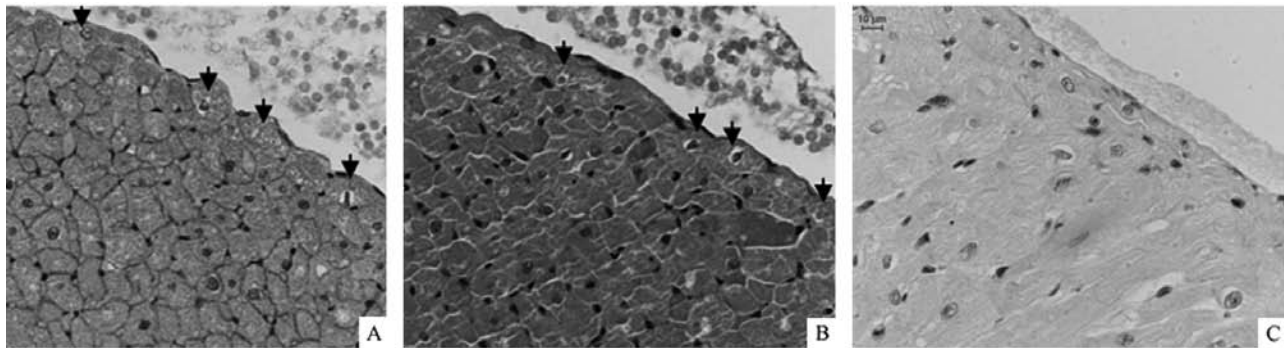


Figure 3. Distribution of TRPC1 in Purkinje cells. These sections were contiguous tissue cross-sections. Endocardial layers were shown. Positive signals, brown in color, could be visualized in Purkinje cells (A, black arrows showed Purkinje cells). The cells beneath the endocardium with loose cytoplasmic structure and without any structure around nuclei were Purkinje cells according to HE staining (B, black arrows showed Purkinje cells). No positive signal could be observed in control experiments (C). Scale bar = 10 μm.

and eosin (HE) staining using the tissue cross-sections contiguous to those used for immunohistochemical study (Figure 3). These results indicate a wide distribution of TRPC1 in the rat hearts,

including working cells, Purkinje cells, endothelial cells and smooth muscle cells of coronary arterioles. No positive signal was observed in fibroblasts. Efforts were also made to display the expression

pattern of TRPC1 in skeletal muscle as a positive control. This procedure could overcome the potential for non-specific staining during immunohistochemical experiments. Our results show that the distribution pattern of TRPC1 in cardiomyocytes is similar to that in skeletal muscle. Both plasma and cell membrane were labeled with TRPC1 antibodies, and the membrane had a stronger stain (Figure 2D).

Two sets of negative control experiments were performed: one with antigen (a peptide with the sequence QLYDKGYTSKEQKDC, corresponding to amino acids 557~571 of the TRPC1 protein) preabsorption and the other in the absence of primary antibodies. No signal was observed in the absence of primary antibodies (Figure 2E, F, G, H). Faint signal was occasionally seen in the antigen preabsorption control, which may be due to insufficient preabsorption (Figure 2I). Nevertheless, the immunospecificity of TRPC1 antibody is authentic, given the distinctively different staining between the experimental group (without preabsorption) and the control group (with preabsorption). The blue color in the images results from hematoxylin counterstaining, showing the locations of cell nuclei.

Confocal images of the ventricular and atrial myocytes stained with anti-TRPC1 antibody showed the cell membrane and plasma localization of TRPC1. Alexa Fluor 488 phalloidin staining showed regular transverse striations of the I bands. We also observed a clear transverse-striation pattern of TRPC1 distribution parallel to and close to the striation of the F-actin stained by phalloidin, consistent with transverse-tubular localization in the ventricular cell (Figure 4), whereas there was no such distribution in the atrial cell which lacked T-tubules.

Both RT-PCR and immunohistochemical experiments were independently repeated at least six times and all results from each repetition were consistent.

Discussion

Recently, endogenous TRPC expression (and in some cases the related protein) have been described in a variety of cell types, including vascular endothelial cells (Antoniotti *et al.*, 2002), smooth muscle cells (Yip *et al.*, 2004), and specif-

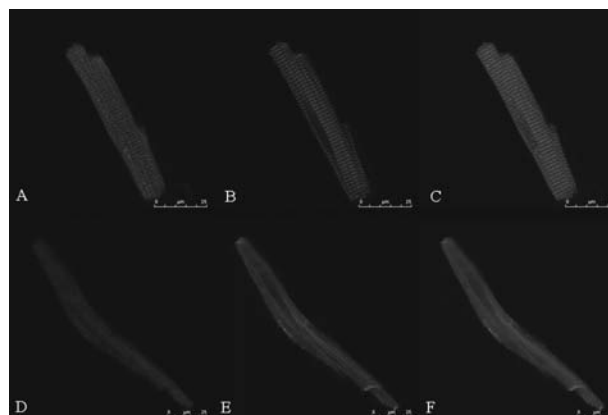


Figure 4. Localization of TRPC1 in rat cardiomyocytes shown by confocal images. Cardiac myocytes were double stained by anti-TRPC1 antibody (A and D) and phalloidin (B and E). Panels C and F show a merged image of panel A/B and D/E respectively, where TRPC1 is colored in red and phalloidin in green. The transverse striation of actin filaments can be seen both in the ventricular myocytes (B) and the atrial myocytes (E). Note that TRPC1 in the ventricular myocyte (A) are parallel to and close to transverse striation of actin filaments, suggesting that they are located at T-tubules while TRPC1 in the atrial myocytes (D) do not show the striation-like distribution. Scale bar =25 μ m.

ic type of nervous system cells (Riccio *et al.*, 2002). Evidence is accumulating that channels of the TRP superfamily play sensory roles in a wide variety of receptor cells, including mechanoreceptor cells (Lin and Corey, 2005). The transduction mechanisms linking stretch and downstream events have not been fully explored, but in most cell types mechanotransduction is mediated by integrin signaling and stretch-activated cation influx (Iqbal and Zaidi, 2005; Shaw and Xu, 2003). Recent reports suggest that proteins of the TRP superfamily form mechanosensitive cation channels (Corey *et al.*, 2004; Maroto *et al.*, 2005). The rise of intracellular calcium in cardiac myocytes and vascular smooth muscle cells may be mediated also via stretch-activated channels (Calaghan *et al.*, 2003; Liao *et al.*, 2003; Zou *et al.*, 2002) besides release of intracellular calcium stores and influxes through L-type cation channel and sodium-calcium exchanger.

The heart is not only a pump but also a mechanosensory system. We propose that the transduction of the stretch signal involves alteration of potential and intracellular calcium signaling caused by the activation of SACCs in heart cells. It is reasonable to believe that TRP channels, as cellular sensors, may play an important role in this process. As a SACC, TRPC1 functions

as an element of a mixed cationic Ca²⁺-permeable channel, and the activity of TRPC1 may contribute to cardiac MEF. To provide morphological proof in support of this hypothesis, we investigated the expression and distribution of TRPC1 in the rat hearts. The results showed that mRNA for TRPC1 was detected in both the atria and the ventricles. The immunohistochemical study showed that the TRPC1 protein is widely expressed in working cardiomyocytes, Purkinje cells, endothelial cells and smooth muscle cells of coronary arterioles, suggesting that TRPC1 plays an important role in the rat hearts. The immunofluorescence study revealed a relatively uniform distribution of TRPC1 in the surface sarcolemma and T-tubule membrane of ventricular myocytes. There is no transverse-striation pattern of TRPC1 in atrial myocytes in accordance with a lack of T-tubules.

Recently it was reported that TRPC1 knockout mouse showed no obvious phenotype, especially store-operated calcium entry in vascular smooth muscle cells (Dietrich *et al.*, 2007). One possible speculation may be the compensatory upregulation of other channels with similar function, which was reported in a study on rats (Selli *et al.*, 2009). Further analysis in different tissues and species should be rewarding. The TRP channels are presumed to be homo- or heterotetramers (Hofmann *et al.*, 2002). The heterologous expression pattern of TRPC1 with other endogenous TRP channels in native cells remains to be determined. Functions of TRPC1 may also be associated with the diversity of channel complexes formed between different isoforms/splice variants and cell-specifically expressed adaptor/signalling proteins. In addition, since the discovery of the TRP channel superfamily, many studies have shown that the TRP superfamily translocate into the plasma membrane upon stimulation (Ambudkar, 2007; Bezzerides *et al.*, 2004; Cayouette and Boulay, 2007) and there is substantial evidence that mechanical stimulation facilitates the membrane trafficking of TRP channels (Inoue *et al.*, 2009). The exact mechanism by which TRP channels insert into the plasma membrane is unknown. Since TRPC1 trafficking to the plasma membrane as well as its retention depends on so many factors, it is unclear whether differences in any of these factors can account for the observed discrepancies concerning the issue of channel pheno-

types (Gottlieb *et al.*, 2008; Maroto *et al.*, 2005).

The present study has clearly and thoroughly shown the expression and localization pattern of TRPC1 in rat hearts in detail and may provide useful data for the future investigations on the functional properties and mechanosensitivity of TRPC1 in rat hearts. The factors involved in regulating TRPC1 expression and trafficking as well as the physiological and pathophysiological functions of TRPC1 channel in its native environment are worthy of further study.

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