

# Unusual localization and translocation of TRPV4 protein in cultured ventricular myocytes of the neonatal rat

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## Abstract

TRPV4 protein forms a Ca<sup>2+</sup>-permeable channel that is sensitive to osmotic and mechanical stimuli and responds to warm temperatures, and expresses widely in various kinds of tissues. As for cardiac myocytes, TRPV4 has been detected only at the mRNA level and there were few reports about subcellular localization of the protein. The purpose of the present study was to investigate the expression profile of TRPV4 protein in cultured neonatal rat ventricular myocytes. Using Western blots, immunofluorescence, confocal microscopy and immuno-electron microscopy, we have shown that TRPV4 protein was predominantly located in the nucleus of cultured neonatal myocytes. Furthermore, cardiac myocytes responded to hypotonic stimulation by translocating TRPV4 protein out of the nucleus. The significance and mechanism concerning the unusual distribution and translocation of TRPV4 protein in cardiac myocytes remain to be clarified.

## Introduction

The transient receptor potential (TRP) channels have attracted increasing interest since the first member was found in a *Drosophila* mutant.<sup>1</sup> Most of the TRP members are nonselective cation channels. The striking features of the TRP superfamily are the functional diversity and almost ubiquitous expression. While most TRP proteins are assembled into the sarcolemma to function, some TRP members may play a role in additional locations besides the cell membrane; for example, TRPP2<sup>2,3</sup> and TRPV4<sup>4</sup> may also be located in cell organelles (the endoplasmic reticulum and Golgi apparatus) as Ca<sup>2+</sup> releasing channels. In addition, TRPML1 to ML3 are thought to be involved in proton-leak channels of intracellular endosomes and lysosomes.<sup>5</sup>

It has been reported that TRPV1, V2 and V4,<sup>6-8</sup> TRPC1, C3 to C7,<sup>9-11</sup> TRPM4 and M5<sup>12,13</sup> and

TRPP2<sup>8,14</sup> are expressed in cardiac myocytes. Accumulating evidence has also suggested that many TRP members are involved in intracellular Ca<sup>2+</sup> regulation, cardiac hypertrophy and arrhythmias. So far, there are only very limited data that TRPV4 mRNA is expressed in adult cardiac muscles.<sup>8</sup> Furthermore, there are no data about TRPV4 protein expression profile in cardiac myocytes. In the present study we reported our discovery that TRPV4 protein was unusually and predominantly localized in the nucleus of the cultured neonatal rat ventricular myocytes. Furthermore, TRPV4 was translocated out of the nucleus upon hypotonic stimulation.

## Materials and Methods

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Capital Medical University, Beijing, China, and were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission, China, 1988).

## Cell culture

Primary cultures of ventricular myocytes were prepared from 1-day-old Sprague-Dawley rats according to the method of Simpson.<sup>15</sup> In brief, the ventricular tissue was digested by 84 U/mL collagenase type II (Worthington Biochemical, Lakewood Township, NJ, USA) and 50 mg/mL trypsin (Gibco, Carlsbad, CA, USA) at 37°C and then the cell suspension was centrifuged (1000×g, 10 min). This digestion course was repeated 10-12 times. The harvested isolated ventricular myocytes were plated on 60 mm dishes at a field density of 6×10<sup>5</sup>-7×10<sup>5</sup> cells/cm<sup>2</sup> and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone, Adelaide, Australia) and 10 mM 5'-BrDU (Roche, Grenzach-Wyhlen, Germany) with 5% CO<sub>2</sub> at 37°C. The cells cultured for 3-6 days were used for this study.

## Immunofluorescence

The ventricular myocytes cultured on coverslips were rinsed three times with cold phosphate buffer saline (PBS) and fixed in 4% paraformaldehyde solution for 15 min. The cells were then permeabilized with 0.1% Triton X-100 in PBS, and treated with 3% H<sub>2</sub>O<sub>2</sub> in absolute methanol. Normal goat serum (10% in PBS) was used to block endogenous biotin. The cells were incubated with the anti-TRPV4 antibody (1:100 dilution, Alomone Labs Ltd., Jerusalem, Israel) at 4°C overnight, and then

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incubated with goat anti-rabbit IgG secondary antibody conjugated with tetramethyl rhodamine isothiocyanate (TRITC) (1:100 dilution, ZSGB-BIO, Beijing, China) for 1 h. The coverslips were then mounted on slides with 90% glycerol in PBS. The same labeling protocol was also used for freshly isolated neonatal and adult ventricular myocytes. Fluorescence signals were observed under a confocal fluorescence microscope with a 40× or 63× objective lens (LAS-AF-TCS SP5, Leica, Solms, Germany).

## Immunohistochemistry

Immunoreactivity in the neonatal and adult rat ventricles was tested using avidin-biotin-peroxidase reactions. Tissue paraffin sections of 3 μm were routinely prepared. After blocking the endogenous biotin with normal goat serum, sections were incubated at 4°C overnight with rabbit anti-rat TRPV4 primary antibody (1:100 dilution, Alomone Labs Ltd.). 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, MO, USA) as a substrate, and sections of the adult ventricle were counterstained with hematoxylin to show nuclei. Images were visualized using an optical microscope (Vanox-T, Olympus, Tokyo, Japan) with a 40× objective lens, and were acquired using an Olympus DP70 camera as well as DP Controller software version 1.2.

## Immuno-electron microscopy

Cultured ventricular myocytes on coverslips were rinsed with PBS, fixed for 2 h in the fixative containing 0.3% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate buffer (PB) and postfixed for additional 2 h in 4% paraformaldehyde in PB. Before immunolabeling of TRPV4 proteins, the myocytes were penetrated by 0.3% Triton X-100 for 20 min and blocked by 6% fresh goat serum in 0.01M PBS. The myocytes were then incubated with the primary (1:1000 dilution, Alomone Labs Ltd.) and secondary antibodies (Ultra-small gold reagents of goat-anti-rabbit IgG, 1:50 dilution, Aurion, Wageningen, The Netherlands). The cells were fixed with glutaraldehyde (2%) followed by a 2-h sliver enhancement process (RGent SE-EM, Aurion) and then a 2-h fixation with 1% osmic acid. Subsequently, the cells were dehydrated step by step. After permeation (for 4 h) and polymerization (37°C overnight and 60°C for 48 h), ultra-thin sections (60 nm) were mounted on electron microscope grids. The grids were dyed by lead nitrate (for 20 min) and uranyl acetate (for 30 min), and the immunolabeling were examined with a JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

## RT-PCR and real-time PCR

Total RNA was extracted with an RNeasy kit (Invitrogen, Carlsbad, CA, USA) from cultured neonatal ventricular myocytes and adult kidney (positive control) of the SD rat. The specific forward and reverse primers for rat TRPV4 were 5'-CCCCGTGGTCTTCATTCT-3' and 5'-CATCTGTGCTGAGTTCTTGT-3' and those for  $\beta$ -actin were 5'-AAGATGACCCAGATCATGTT-3' and 5'-TTAATGTCACGCACGATTTC-3', respectively. PCR products (expected fragment sizes: TRPV4, 446 bp;  $\beta$ -actin, 287 bp) were analyzed on a 1.5% agarose gel by electrophoresis and visualized with ethidium bromide. The authenticity of amplified PCR products was verified using an ABI PRISM DNA sequencing system (Perkin Elmer, Boston, MA, USA).

Real-time PCR was performed according to a comparative quantitative analysis (Quick protocol of Mxpro™ QPCR software for Mx3000P system; Stratagene, La Jolla, CA, USA) in a total volume of 20  $\mu$ L using 96-well microwell plates. A 45-cycle PCR program was carried out according to the following protocol: pre-denaturation for 10 min at 95°C, denaturation for 30 sec at 95°C, annealing for 1 min at 57°C and elongation for 1 min at 72°C. Forward and reverse primers, specific for rat TRPV4, were 5'-CAAGTGGCGTAAGTTCGG-3' and 5'-CCTGTGAGGAGCGTGATG-3', respectively. These primers yielded a 180-bp PCR product. Primers for  $\beta$ -actin were

the same as those used in the RT-PCR experiments.

## Western blots

Total protein was extracted from the cultured neonatal and the freshly isolated adult ventricular myocytes according to the reference.<sup>16</sup> The cells were harvested in buffer A that containing (in mM) 50 Tris-HCl (pH 7.5), 50 NaF, 2 EDTA, 2 EGTA, 0.1 Na orthovanadate and 1 DTT with 2% SDS and 15% protease inhibitor cocktail (Roche). Homogenates were centrifuged at 33,000 $\times$ g for 30 min at 4°C. The supernatant (total proteins) was transferred and stored at -80°C.

Nuclear proteins were extracted by using a modified protocol (<http://www.ualberta.ca/~olsonlab>). In brief, the cultured neonatal ventricular myocytes were collected in buffer B containing (in mM) 10 HEPES (pH 7.9 with KOH), 10 KCl, 1.5 MgCl<sub>2</sub>, 0.1 EDTA, 0.1 EGTA, 1 DTT and 15% protease inhibitor cocktail. The samples were placed on ice for 15 min after being disrupted by brief sonication and then exposed to 0.5% NP-40 followed by incubation on ice for 30 min and centrifugation at 6000 $\times$ g for 6 min at 4°C. The sediment was then resuspended in buffer C containing (in mM) 20 HEPES (pH 7.9), 420 NaCl, 1.5 MgCl<sub>2</sub>, 0.1 EDTA, 0.1 EGTA and 1 DTT with 25% glycerol and 15% protease inhibitor cocktail. The samples were centrifuged again at 33,000 $\times$ g for 30 min at 4°C after being placed on ice for 30 min. The supernatant (nuclear proteins) was transferred and stored at -80°C.

Protein samples from cardiomyocytes (30  $\mu$ g or 50  $\mu$ g proteins) were separated by electrophoresis on an 8% polyacrylamide gel (for nucleus protein separation, a 12% gel was used) and transferred onto a cellulose acetate membrane. Nonspecific binding sites were blocked with 10% skim milk in Tris-buffered saline solution (TBS) (2 h at room temperature). The membrane was incubated with polyclonal anti-TRPV4 antibody (1:500 dilution, Alomone Labs Ltd.) in TBS solution with 0.05% Tween-20 and 10% defatted milk powder (TBST-milk) at 4°C overnight with agitation. The antibody is directed specifically against a peptide of CDGHQQGYAP-KWRAEDAPL, corresponding to amino acid residues 853-871 of rat TRPV4 (accession Q9ERZ8). After being washed, the membranes were then treated with IRDye™ 700 conjugated affinity purified anti-rabbit secondary IgG for 1 h at room temperature, followed by three washes with TBST and two washes with TBS alone. Fluorescent bands were visualized using an LI-COR Odyssey infrared double-fluorescence imaging system (LI-COR Inc., Lincoln, NE, USA).

To compare the expression of TRPV4 before and after hypotonic stimulation both in the

whole cell and the nucleus, we used  $\beta$ -actin as an internal loading control. It has been accepted widespread that  $\beta$ -actin is an indispensable constituent of nuclear proteins.<sup>17</sup> The expression of  $\beta$ -actin was also demonstrated to be stable during exposure to hypotonicity.<sup>18</sup>

## Solution

The isotonic solution (300 mOsm/L) contained (in mM) 100 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 90 D-mannitol, and was adjusted to pH 7.4 with NaOH. The hypotonic medium (210 mOsm/L) was produced by omitting D-mannitol from the isotonic solution. The osmolality of the solution was measured with an osmometer (Fiske 110, Fiske Associates, Norwood, MA, USA) at 0°C.

## Data analysis

Data were presented as the mean value  $\pm$  SEM. Student's paired and unpaired *t*-tests were performed by GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA, USA). Values of *P* < 0.05 were considered statistically significant.

## Results

### Localization of TRPV4 protein in cardiac myocytes

Immunochemical analysis of TRPV4 protein was performed on ventricular myocytes. In freshly isolated neonatal myocytes, the TRPV4 immunological signal (TRPV4-TRITC, red) was mainly localized around the nucleus (Figure 1A). DAPI (blue) was used to stain the nucleus. In contrast, the immunological signal for TRPV4 was very strong in the nucleus of cultured neonatal myocytes (Figure 1B-1), while the stain outside the nucleus was weak. Notably, TRPV4 immunoreactivity distribution in freshly isolated adult ventricular myocytes was similar to that in cultured neonatal cells (Figure 1C). In addition, we confirmed that TRPV4 protein was also mainly localized in the nucleus of neonatal and adult ventricular myocytes by immunohistochemistry (Figure 1F,G). To exclude the possibility of a pseudo-positive reaction for the fluorescence signal in the nucleus, a blank control test without TRPV4 antibody was performed and a negative result was confirmed (Figure 1D). In addition, the positive signals for TRPV4 protein in the cultured ventricular myocytes disappeared in the antibody absorption test, demonstrating that the antibody was specific (Figure 1E).

## Hypotonically induced translocation of TRPV4 protein in cultured neonatal ventricular myocytes

It has been reported that TRPV4 channel is activated by cellular swelling<sup>19</sup> and translocation of TRPV4 protein in endothelial cells can occur in response to mechanical stimulations.<sup>4</sup> To test the possibility of TRPV4 translocation in cultured neonatal ventricular myocytes when challenged by hypotonic stimulation (210 mOsm/L, 45 min), the distribution of TRPV4 protein before and after hypotonic exposure were compared.

Figure 2A shows a strong immunoreaction in the nuclear area for TRPV4 protein and a faint immunological signal outside the nucleus in the isotonic solution. However, after a 45-min hypotonic exposure, the fluorescence in the nuclear zone became much weaker while the extranuclear TRPV4 signal was enhanced (Figure 2B). Immuno-electron microscopy was used to further investigate the subcellular localization of TRPV4 protein in cultured ventricular myocytes before and after hypotonic treatment. TRPV4 immunoreaction clearly focused on the nuclear zone and less existed outside the nucleus (Figure 2C). After hypotonic stimulation (Figure 2D), the quantity of colloid gold granules in the nuclear area was greatly decreased, while immunogold labeling outside the nucleus was increased. These results reinforce the observation that hypotonic stimulation could trigger an outward translocation of TRPV4 protein from the nucleus.

RT-PCR analysis was performed to ascertain the expression of TRPV4 in ventricular myocytes. As shown in Figure 3 A, mRNA for TRPV4 was detected in neonatal cultured ventricular myocytes and adult renal tissue (positive control) of the SD rat. The identity of the PCR product was further verified by sequencing (*data not shown*). Moreover, real-time PCR analysis was carried out to quantify the change of TRPV4 mRNA in neonatal cultured myocytes after hypotonic stimulation. Figure 3B showed that TRPV4 mRNA was not altered by hypotonic challenge ( $P>0.05$ ,  $n=12$ ).

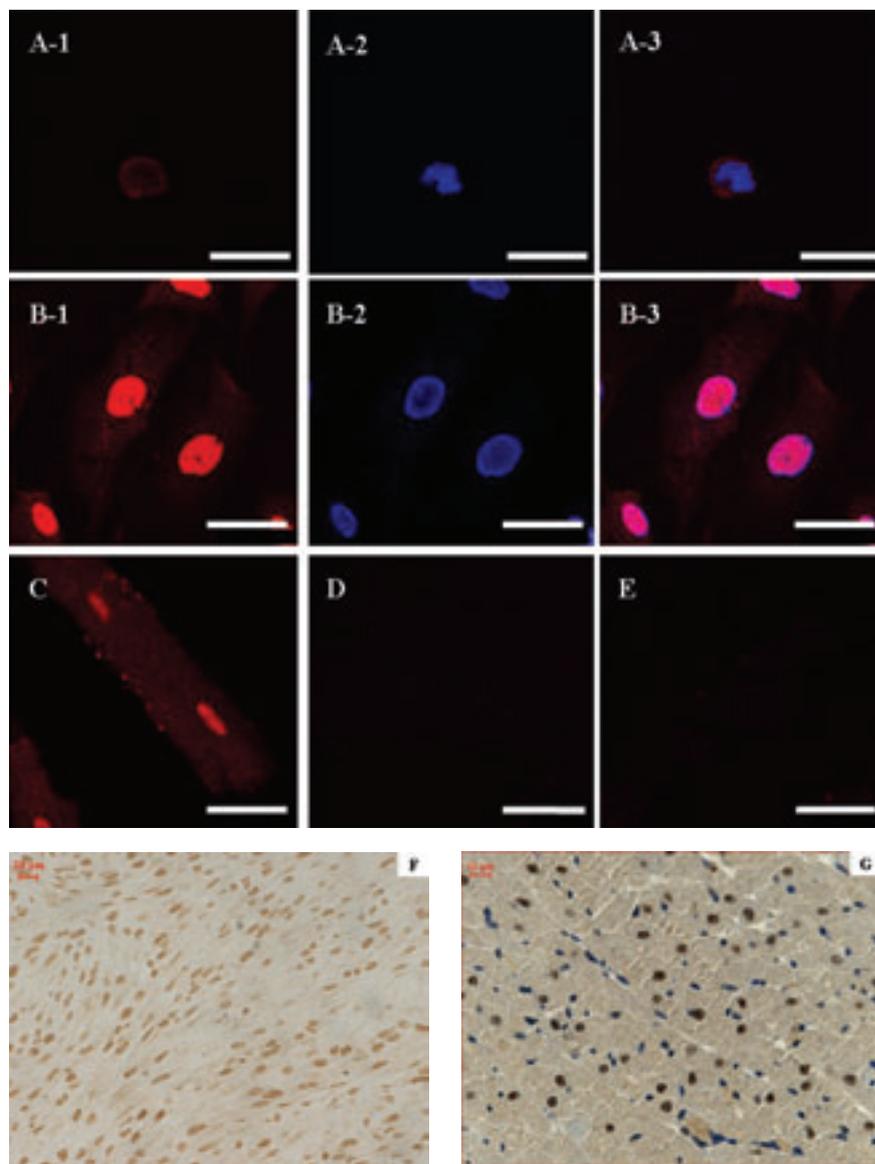
To further examine the expression and localization of TRPV4 at protein level, Western blot analyses were performed on the whole and the nucleus of cultured neonatal ventricular myocytes. The same two bands at ~70 and ~90 kDa were recognized with anti-TRPV4 antibody in the freshly isolated adult (Figure 3C) and cultured neonatal ventricular myocytes (Figure 3D), and also in the nucleus fraction of the latter (Figure 3E). Statistical analyses indicated that the quantity of TRPV4 protein in the whole cultured

neonatal ventricular cell was not changed during the exposure to hypotonic solution (Figure 3 D,F;  $P>0.05$ ;  $n=5$ ), however, that in the nucleus fraction was significantly decreased (Figure 3 E,F;  $P<0.05$ ;  $n=15$ ). These results conformed our discovery in the immunocytochemical study that hypotonic stimulation resulted in translocation of TRPV4 protein outward from the nucleus in cultured neonatal ventricular myocytes.

## Discussion

### Unusual localization of TRPV4 protein in cultured ventricular myocytes of the neonatal rat

In this study, we showed that TRPV4 protein was expressed in ventricular myocytes of the neonatal rat (Figures 1, 2 and 3). TRPV4 pro-



**Figure 1.** Localization of TRPV4 protein in cardiac myocytes. A, B) Confocal images of freshly isolated (A1-3, scale bar: 15  $\mu$ m) and cultured neonatal ventricular myocytes (B1-3, scale bar: 25  $\mu$ m) labeled with anti-TRPV4 antibody (A1 and B1) and DAPI (A2 and B2), respectively, and the merged images (A3 and B3). The freshly isolated neonatal ventricular myocyte was small and round. C) Confocal images of freshly isolated adult rat ventricular myocytes labeled with anti-TRPV4 antibody (scale bar: 25  $\mu$ m). D) Confocal image of the cultured ventricular myocytes in blank control (without TRPV4 antibody). E) Confocal image of the cultured ventricular myocytes in absorption test, in which the anti-TRPV4 antibody was preincubated with the peptide antigen. F, G) Immunolabeling TRPV4 protein in sections of the neonatal and adult ventricles.

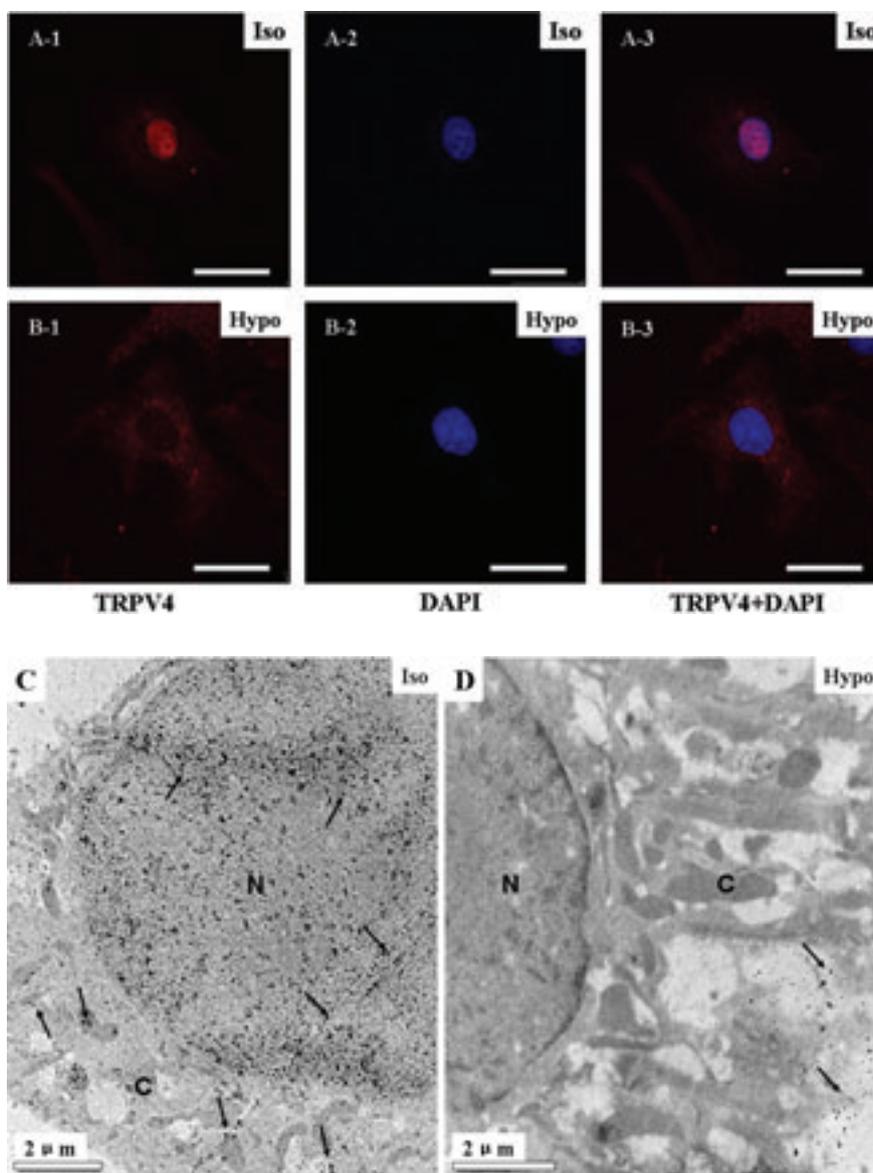
tein has been thought to be inlaid in the cell membrane and to be assembled into ion channels. However, surprisingly, we found that the immunofluorescent signal of TRPV4 was extremely strong in the nucleus and seemed extraordinary. To confirm the antibody specificity and the authenticity of this phenomenon, four different experiments were performed. First, two types of negative control trials, blank and antibody absorption tests were carried out in immunofluorescence experiments. In both negative control experiments, positive signals could hardly be detected, demonstrating that the antibody was specific. Second, in Western blot analyses, the TRPV4 bands were not identified in the absorption tests (Figure 3C), which also verified the specificity of the TRPV4 antibody. Third, by means of immuno-electron microscopy, we confirmed that colloidal gold particles were localized in the cultured ventricular myocyte with a distinctly higher density in the nucleus (Figure 2C). Fourth, in the Western blot analyses on TRPV4 protein from the whole cell and nucleus, the same molecular mass of the bands confirmed the expression profile of TRPV4 protein (Figure 3 D,E). Taken together, these results indicated that TRPV4 protein was located predominantly in the nucleus of cultured neonatal rat ventricular myocytes.

Human TRPV4 consists of 871 amino acids and has five variants including TRPV4-A (full-length form), TRPV4-B (lacking exon 7), TRPV4-C (lacking exon 5), TRPV4-D (short deletion inside exon 2) and TRPV4-E (lacking  $\Delta 237-284$  and  $\Delta 384-444$  amino acids).<sup>18</sup> The immunoreactive protein bands have apparent molecular masses of 96, 100 and 110 kDa for TRPV4-A and TRPV4-D, 91 and 96 kDa for TRPV4-B, 92 and 97 kDa for TRPV4-C, and 86 and 90 kDa for TRPV4-E, respectively. The different bands for a particular variant were shown to be caused by glycosylation.<sup>20</sup> In the mouse, wild type TRPV4 protein has a predicted molecular weight of 98 kDa.<sup>21</sup> It was reported that anti-TRPV4 antibody detected two bands at ~98 and ~107 kDa in the kidney extract obtained from mice<sup>22</sup> and in DRG neurons isolated from adult male rats.<sup>23</sup> The isoform of ~107 kDa was considered as a glycosylated protein. Furthermore, Liedtke and Friedman<sup>24</sup> detected a shorter isoform of 75 kDa, which was thought to be a yet undescribed splice variant, in addition to the 107 kDa protein for TRPV4 in mouse kidney extract. In the present study, two similar bands of TRPV4 protein with molecular weights of ~70 and ~90 kDa were detected in adult and neonatal cardiomyocytes (Figure 3 C,D).

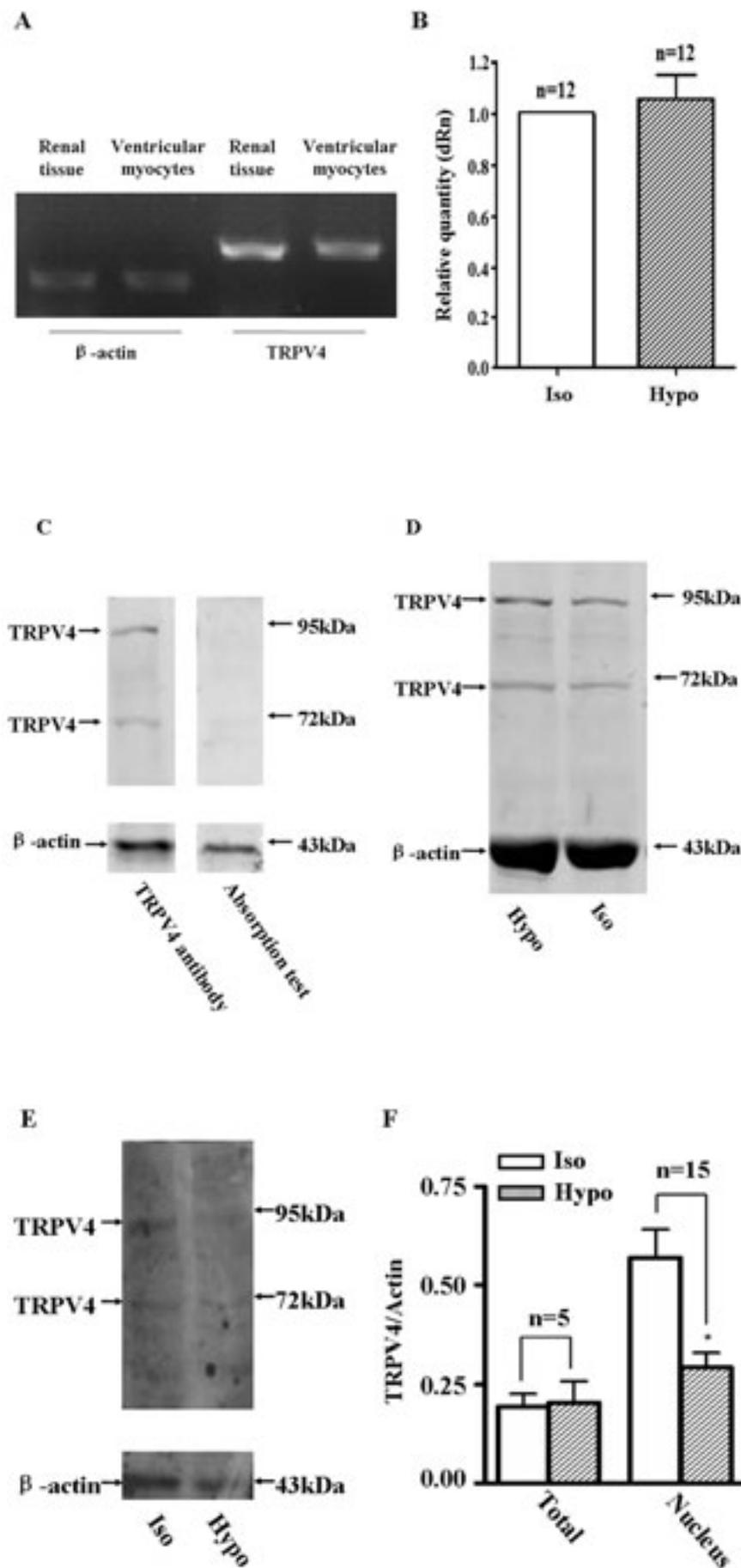
Some reports have described that there are bright immunological signals in the nucleus for several types of channel proteins. TRPV4 immunoreactivity has been localized at the

plasma membrane and in the nuclear and perinuclear areas of primary afferents isolated from mouse dorsal root ganglia<sup>25</sup> and the organum vasculosum of the lamina terminalis.<sup>24</sup> In many substantia nigra neurons, TRPV1 protein is stained as a punctuate-like pattern in the cytoplasm as well as the nucleus.<sup>26</sup> Glazebrook *et al.*<sup>27</sup> showed that TRPC6 immuno-signals occur in the nucleus of rat nodose ganglion neurons using the corresponding antibody of three different sources.

Furthermore, TASK1 immunofluorescence has been observed throughout plasmalemmal and intracellular locations, and TREK1 immuno-signals are localized in all three cellular fractions of myometrial cells.<sup>28</sup> We have also noticed a figure in a paper<sup>29</sup> showing bright immunostaining for TREK1 both inside and outside the nucleus of the adult rat ventricular muscle and the transfected and cultured COS cell. Thus, it seems not uncommon that certain types of channel proteins could move into the



**Figure 2.** Effects of hypotonicity on the distribution of TRPV4 in ventricular myocytes. Iso and Hypo: isotonic and hypotonic bath solutions, respectively. A, B) Immunofluorescence localization of TRPV4 protein in cultured ventricular myocytes before (A) and after (B) hypotonic stimulation (scale bar: 25  $\mu$ m). The myocytes were doubly labeled for TRPV4 protein (A-1, B-1) and the nucleus (A-2, B-2) as did as in Figure 1. A-3 and B-3 were correspondingly overlaid images. C, D) Immunoreactivity of TRPV4 protein detected by immuno-electron microscopy in cultured ventricular myocytes before (C) and after (D) hypotonic stimulation. N, nucleus; C, cytoplasm; arrows indicate the colloidal gold granules.



nucleus. There is evidence that multiple receptor tyrosine kinases, one type of membrane integrate proteins with a single transmembrane domain, could traffic to the nucleus in the full-length form.<sup>30,31</sup> However, since TRPV4 protein contains six hydrophobic transmembrane-spanning domains, we guess that TRPV4 molecule would be more restrained by the lipid bilayer membrane when being transported into the nucleus. More investigations would be imperative for clarifying the mechanism of TRPV4 protein trafficking.

Polycystin-1, a TRPP2 related protein, can undergo proteolysis and release its carboxy-terminal tail, which translocates to the nucleus and activates transcription factor AP1.<sup>32</sup> A carboxy-terminal fragment of connexin 43, a subunit of gap junction channel, has been localized in nuclei of the cardiomyocyte and HeLa cell and shown a non-channel function (to inhibit HeLa cell growth).<sup>33</sup> It is possible that the immunofluorescence in our study might have been a reaction between the anti-TRPV4 antibody and a TRPV4 peptide segment if it was cleaved proteolytically from the protein and transported into the nucleus. However, Western blot analyses on the molecular weight did not support an immunocomplex formed by a short TRPV4 peptide segment with its antibody. It also seems unlikely that a different protein with the same molecular weight could bind with the anti-TRPV4 antibody.

**Figure 3.** Hypotonically induced translocation of TRPV4 protein in cultured ventricular myocytes. **A)** The TRPV4 mRNA transcript was detected in adult renal tissues and cultured neonatal ventricular myocytes by RT-PCR amplification. **B)** Quantification of TRPV4 mRNA by real-time PCR for cultured ventricular myocytes in isotonic bath solution (Iso) and after hypotonic stimulation (Hypo). There were no significant differences at the mRNA levels between the two groups. **C)** Western blot analysis on the total TRPV4 protein of the freshly isolated adult ventricular myocytes and the corresponding absorption test. **D)** Western blot analysis on the total TRPV4 protein of cultured neonatal ventricular myocytes before and after exposure to hypotonic stimulation. **E)** Western blot analysis on TRPV4 protein in the nucleus fraction before and after hypotonic stimulation. **F)** Total and nuclear TRPV4 protein under isotonic and hypotonic conditions. The longitudinal coordinate stands for the relative ratio of TRPV4 fluorescent value contrast to  $\beta$ -actin fluorescent value (\* $P < 0.05$ ).

## Translocation of TRPV4 protein in cultured ventricular myocytes

It is common for particular proteins, *e.g.*, channels, to be translocated upon proper stimulations. Studies have demonstrated that insulin and insulin-like growth factor-I (IGF-I) enhance TRPV1-mediated membrane currents in heterologous expression systems and cultured dorsal root ganglion neurons. The enhancement of the membrane current results from both the increased sensitivity of TRPV1 and translocation of TRPV1 from cytosol to plasma membrane.<sup>34</sup> Recently, Loot *et al.*<sup>4</sup> reported that shear stress could induce translocation of TRPV4 from the Golgi apparatus to the cell membrane in cultured human endothelial cells. Cuajungco *et al.*<sup>35</sup> found that co-expression of TRPV4 and PACSIN 3, a binding protein of TRPV4 and one member of the PACSIN family, increases the ratio of plasma membrane-associated versus cytosolic TRPV4. Moreover, microfilament-associated protein 7 has been implicated in increasing the membrane expression of TRPV4,<sup>36</sup> and kinases of the WNK family have been reported to influence the function and localization of TRPV4.<sup>37</sup> In the present study, TRPV4 protein was shown with unusual distribution profiles, dominant in the perinuclear region in freshly isolated neonatal ventricular myocytes and notable in the nucleus of cultured neonatal and freshly isolated adult ventricular myocytes. More importantly, TRPV4 protein moved out of the nucleus in response to hypotonic stress in cultured myocytes. These results strongly suggested that TRPV4 protein could shuttle into and out of the nucleus.

It has been suggested that TRPV4 can sense diverse physical stimuli and convert them to Ca<sup>2+</sup> signals in various mammalian tissues. Mice lacking the TRPV4 gene have reduced regulation of serum osmolarity and an increased mechanical nociceptive threshold.<sup>24,38,39</sup> Furthermore, TRPV4 functions as a transducer of hypo-osmotic stimuli in primary afferent nociceptors<sup>40</sup> and plays an essential role in taxol-induced nociceptive behavioral responses to mechanical and hypotonic stimulations on the hind paw.<sup>41</sup> All these functions are explained on the basis of its channel identity. However, in the present study, we provided new evidence that TRPV4 protein is located mainly in the nucleus of cultured neonatal ventricular myocytes and that TRPV4 protein was translocated out of the nucleus in response to hypotonic stimulation. This nuclear localization of TRPV4 protein seems not in relation to channel functions. The significance of TRPV4 shuttling in cultured neonatal ventricular myocytes remains to be illuminated.

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