Cloning and Functional Expression of a Novel Splice Variant of Rat TRPC4

Eisaku Satoh; Kyoichi Ono, MD; Feng Xu, MD; Toshihiko Iijima, MD

Transient receptor potential protein 4 (TRPC4) has been identified as a candidate for the capacitative calcium entry (CCE) channels, but its functional role is still controversial. Using a RT-PCR technique, a novel isoform of TRPC4, designated rTRPC4, was isolated. It was nearly identical to full-length rTRPC4 (rTRPC4), except that it lacked 53 nucleotides that correspond to the predicted linker between the second and third transmembrane domain of rTRPC4, and its mRNA was expressed in brain and heart. This splice variant encoded a potential protein of 400 residues that consists of an amino-terminal cytoplasmic domain and 2 transmembrane domains by a frameshift mutation. When rTRPC4 cDNA was transiently transfected to HEK-293 cells, thapsigargin (TG)-induced Ca2+ entry was suppressed significantly. By contrast, expression of rTRPC4 did not affect TG-induced Ca2+ entry. To investigate the subcellular localization, plasmids were constructed with green fluorescence protein (GFP) as an amino-terminal fusion to rTRPC4 variants. GFP-rTRPC4, was localized to the cytoplasm as well as plasma membrane. These results suggest that rTRPC4 may play a modulatory role in CCE channel activity in the brain and heart. (Circ J 2002; 66: 954–958)

Key Words: Capacitative calcium entry; HEK-293 cells; Splice variant; Thapsigargin; Transient receptor potential proteins

In many non-excitable cells, activation of phospholipase C-linked receptors results in a biphasic increase in the intracellular Ca2+ concentration ([Ca2+]i); that is, an initial transient increase because of the release of Ca2+ from intracellular store sites, followed by sustained elevation by Ca2+ influx via plasma membrane. The sustained [Ca2+]i increase, termed capacitative calcium entry (CCE) or store-operated calcium entry (SOCE), plays a crucial role in various cellular function. Several members of the transient receptor potential (TRP) channel superfamily are candidate molecules for these mechanisms, and of these genes, TRPC4 is of particular interest, because an earlier study showed that heterologous expression of TRPC4 augmented CCE in response to store-depletion by receptor stimulation and thapsigargin (TG), a Ca2+-ATPase inhibitor. Although the function of TRPC4 proteins has been extensively studied on CCE by using several measures of Ca2+ entry through the cell membrane, the experimental results differ.

TRPC4 has 6 transmembrane domains (TMD) with both carboxy and amino termini located intracellularly. Several recent studies have shown that the carboxy terminus contributes to the interaction with other cellular proteins implicated for channel activation by contrast, the physiological roles of the amino terminus with ankyrin domains are still unknown. In the present study, we isolated a novel splice variant of TRPC4, designated rTRPC4, from rat brain cDNA. rTRPC4 mRNA lacked 53 nucleotides that corresponded to the predicted linker between TMD2 and TMD3 of rTRPC4, and encoded a potential protein of 400 residues that consists of an amino terminal cytoplasmic domain and 2 transmembrane domains by a frameshift mutation. We transiently transfected rTRPC4 into HEK-293 cells and examined the function of rTRPC4 in CCE, using fluorescent [Ca2+]i measurement.

Methods

Isolation of cDNA Encoding Rat TRPC4 Channel

Rat brain Marathon-Ready cDNA (CLONTECH) was used for cloning by reverse transcriptase-polymerase chain reaction (RT-PCR). The amplification of the coding region of full-length rTRPC4 (rTRPC4) was performed by RT-PCR using a pair of primer, forward (5′-ATGGCTCAGTT-CTATTACAACGAAATGTC-3′) paired with a reverse (5′-TCACAATCTCAGGTCACATAATCCTAG-3′). The polymerase chain reaction (PCR) conditions were 5 initial cycles of 94°C for 15 s and 72°C for 4 min, followed by 5 cycles at 94°C for 15 s, and 70°C for 4 min, with final 25 cycles of 94°C for 15 s and 68°C for 4 min. PCR products were gel-purified, subcloned into a TA-cloning vector (pCR II, Invitrogen). DNA sequencing was performed using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an automated sequencer (ABI-PRISM-310, Applied Biosystems).

RT-PCR Detection of rTRPC4 Variants

To confirm the existence of rTRPC4 variants, rat brain Marathon-Ready cDNA was amplified by PCR using a forward primer (nucleotides 1141–1165 in rTRPC4; 5′-CTGCTAGGCTTCTCAGACATCGA3′) paired with a reverse primer (nucleotides 1525–1549; 5′-GGATGTCCTAGGAGCATTTCTCCCA-3′). The PCR conditions were 30 cycles of 94°C for 30 s and 68°C for 2 min. Rat multiple tissue cDNA (RAT MTC Panel, CLONTECH) was used to detect the distribution of rTRPC4 variants in different rat tissues. The PCR conditions were the same as described before, except that the number of cycles was increased to 35.

(Received May 24, 2002; revised manuscript received July 12, 2002; accepted July 12, 2002)

Department of Pharmacology, Akita University School of Medicine, Akita, Japan

Mailing address: Toshihiko Iijima, MD, PhD, Department of Pharmacology, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. E-mail: tiiijima@med.akita-u.ac.jp
**Cell Culture and Transfection**

HEK-293 cells, obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan, No. JCRB9068), were cultured in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and antibiotic/antimycotic (Invitrogen). For transfection, we used LIPOFECTAMINETM PLUS Reagent (Invitrogen) according to the manufacturer’s instructions. The coding region for rTRPC4 was subcloned into a pRES2-EGFP vector (CLONTECH) and green fluorescence protein (GFP) fluorescence was used as a marker for transfection. Cells were assayed at 20–36 h post-transfection. To visualize subcellular localization of rTRPC4 and rTRPC4, amino-terminal GFP chimeras were generated using amino-terminal GFP fusion vector pQBI25-fC3 (Qbiogene, Illkirch, France).

**Measurement of [Ca2+]i**

Cells were loaded with 5 mol/L fura-2 acetoxyethyl ester (Dojindo Laboratories, Kumamoto, Japan) at 37°C for 60 min in HEPES-buffered saline (HBS) containing (in mmol/L) 136.9 NaCl, 5.4 KCl, 1 CaCl2, 1.0 MgSO4, 11.1 glucose, and 5.0 HEPES, with pH 7.4 adjusted with NaOH. After an additional 30-min incubation at 37°C, cells were rinsed several times with HBS and used for experiments within 3 h. HBS containing 5 mmol/L CaCl2 instead of 1 mmol/L CaCl2 was used as standard bath solution. Ca2+ free solution contained 5 mol/L EGTA in HBS without CaCl2. Carbachol and thapsigargin were purchased from Wako Pure Chemicals (Osaka, Japan). Measurement of [Ca2+]i was performed in a single cell showing GFP fluorescence. The excitation wavelength alternated at 400 Hz between 340 and 380 nm and the emission fluorescence at 500 nm was measured using a spectro-photofluorimeter (CAM-230, Japan Spectroscope Co, Tokyo, Japan). The emission intensities at 340 and 380 nm excitation (F340 and F380) was subtracted from raw signals at each excitation wavelength for each experiment on cell-free parts of the coverslip, was measured at the end of experiments. Autofluorescence, measured at the end of each experiment on cell-free parts of the coverslip, was subtracted from raw signals at each excitation wavelength before calculating the fluorescence ratio, F340/F380. In the present experiments, [Ca2+]i was expressed in the ratio. All experiments were conducted at 37°C.

**Confocal Microscopy**

Cells were cultured for 36–60 h after transfection and were viewed under a Noran Oz confocal laser scanning microscope system (NORAN Instruments Inc, Middleton, WI, USA) equipped with an argon-ion laser using 488 nm excitation/carboxy-terminal region of rTRPC4 clone encoded a known carboxy-terminal splice variant of TRPC4. Because the deficits cause a frameshift, rTRPC4 encodes a potential protein of 400 amino acids that lacks the carboxy-terminal 577 amino acids of rTRPC4 (Fig 1C). As shown in Fig 1A, the region containing the deletion part was amplified with PCR. Specific primers were designed to yield transcripts of 409 bp for rTRPC4 and 356 bp for rTRPC4, respectively. As shown in Fig 1D, both transcripts were expressed in rat brain cDNA. Furthermore, in the RT-PCR assay on the tissue distribution of TRPC4 splice variants, the 409 bp band was widely detected in brain, heart, lung, kidney, testis, and skeletal muscle, whereas rTRPC4 was expressed only in brain and heart (Fig 1E).

**Results**

**Isolation of cDNAs Encoding TRPC4 Channel**

The RT-PCR using the primers for rTRPC4 of rat brain cDNA library revealed 2 visible bands (Fig 1A). The sequence analysis showed that the upper thick band contained 2 transcript of 2,934 bp, 2,881 bp (Fig 1B). The clone of the 2,934 bp encoded a protein consisting of 977 amino acids, which was identical to rat TRPC4 (Gene bank accession No. AF288407) except for 3 amino acid substitutions (D594E, K898Q, and H949R). The 2,881 bp clone had a deficit of 53 bp that correspond to the predicted substitutions (D594E, K898Q, and H949R). The 2,881 bp clone contained a potential protein of 400 amino acids that lacks the carboxy-terminal 577 amino acids of rTRPC4 (Fig 1C). As shown in Fig 1A, we obtained the other transcript of 2,682 bp from the lower thin band. The clone encoded a known carboxy-terminal splice variant of rTRPC4, rTRPC4, which lacked 84 amino acids in the carboxy-terminal region of rTRPC4.

To clarify the existence of rTRPC4, the region containing the deletion part was amplified with PCR. Specific primers were designed to yield transcripts of 409 bp for rTRPC4 and 356 bp for rTRPC4, respectively. As shown in Fig 1D, both transcripts were expressed in rat brain cDNA. Furthermore, in the RT-PCR assay on the tissue distribution of TRPC4 splice variants, the 409 bp band was widely detected in brain, heart, lung, kidney, testis, and skeletal muscle, whereas rTRPC4 was expressed only in brain and heart (Fig 1E).

**Functional Role of rTRPC4 on CCE in HEK-293 Cells**

To investigate the functional role of rTRPC4 in CCE,
rTRPC4 was transfected transiently to HEK-293 cells, and [Ca\(^{2+}\)] was measured using the fura 2 loading method. Typical traces from HEK-293 cells transfected with rTRPC4 (A), rTRPC4 (B), or expression vector alone (C) are shown in Fig. 2. In the Ca\(^{2+}\)-free extracellular solution, 100 nmol/L TG caused a similar transient increase of [Ca\(^{2+}\)], but it was apparently suppressed in rTRPC4-transfected cells. The mean of maximal [Ca\(^{2+}\)] changes was summarized in Fig. 2D. In rTRPC4-transfected cells, the sustained [Ca\(^{2+}\)] rise was 0.20±0.05 (n=7) and was significantly smaller than the control and rTRPC4-transfected cells (0.55±0.07, n=11 and 0.52±0.10, n=9, respectively). In our experiments, rTRPC4-expression had no effect on the TG-induced Ca\(^{2+}\) influx.

Then, we observed the [Ca\(^{2+}\)] response to carbachol (CCh), which is known to elicit CCE through store depletion by activation of the inositol-1,4,5-triphosphate (IP\(_3\)) receptor, in these cells (Fig. 3). CCh at 100 nmol/L transiently increased [Ca\(^{2+}\)], and caused a sustained increase of [Ca\(^{2+}\)]; after re-addition of 5 mmol/L Ca\(^{2+}\). In the control cells, the transient [Ca\(^{2+}\)] rise was 0.57±0.05 (n=14) and was significantly smaller than the control and rTRPC4-transfected cells (0.55±0.07, n=11 and 0.52±0.10, n=9, respectively). In our experiments, rTRPC4-expression had no effect on the CCh-induced [Ca\(^{2+}\)] influx.

Localization of GFP-rTRPC4 or GFP-rTRPC4 Fusion Protein in HEK-293 Cells
Because rTRPC4 lacked a large portion of the transmembrane domain, we fused the amino terminus of
either rTRPC4 or rTRPC4 with GFP (GFP-rTRPC4 or GFP-rTRPC4) to clarify the subcellular localization of rTRPC4. The resulting cDNA was transfected into HEK-293 cells, and analyzed by a confocal laser scanning fluorescence microscope. Strong fluorescence of GFP-rTRPC4 was recognized at the cell periphery (Fig 4A). By contrast, GFP fluorescence diffused throughout the cytoplasm (Fig 4C). These findings suggested that rTRPC4 was expressed in the plasma membrane. Unlike GFP-rTRPC4, the fluorescence of GFP-rTRPC4 was localized to the plasma membrane and part of the cytoplasm (Fig 4B).

Discussion

Using RT-PCR strategies, we identified a novel splice variant of rTRPC4, rTRPC4, which encodes a potential protein of 400 residues that consists of an amino-terminal cytoplasmic and 2 transmembrane domains. Its mRNA was expressed in brain and heart. Expression of rTRPC4 suppressed the TG-induced [Ca2+]i response in HEK-293 cells, whereas rTRPC4-expression was unaffected. The suppression is not because of a possible change in the Ca2+ content in the store sites, because the TG-induced transient Ca2+ rise in Ca2+-free solution was not affected by the expression of rTRPC4. The present results indicate that the lack of the C-terminus in rTRPC4 accounts for the inhibitory effect on TG-induced Ca2+ entry.

A recent study has reported the expression of TRPC1, TRPC3, TRPC4 and TRPC6 mRNA in HEK-293 cells, and that TG-induced endogenous Ba2+ entry in HEK-293 cells is reduced by transfection of antisense constructs of human TRPC4-transfected HEK-293 cells.8 However, several studies have shown that TRPC4 is involved in CCE or receptor-operated Ca2+ entry,4,5,7 From the evidence that GFP-rTRPC4 protein is localized to the plasma membrane, it is not conceivable that the functional loss of rTRPC4 is because of impaired translation or protein trafficking. Thus, it appears that rTRPC4 alone cannot reconstitute CCE channels in HEK-293 cells. The discrepancy among various studies indicates that functional expression might require interaction between rTRPC4 and other unidentified molecules.

In conclusion, this study reports the cloning of a splice variant of rat TRPC4 and its suppressive effect on TG-induced Ca2+ entry in HEK-293 cells. This mRNA is expressed in brain and heart. Although the physiological significance of rTRPC4 is still unclear, it may play a modulatory role in regulating CCE channel activity in these tissues.

Acknowledgments

This work has been supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, and in part by Japan Heart Foundation and IBM Japan Research Grant 1999. We also thank Mrs Hitomi Meguro for her technical assistance and Mrs Miyako Ooyama for her secretarial support.

References