Cell Calcium 51 (2012) 186–193

Contents lists available at SciVerse ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Translocation of calcium-permeable TRPV2 channel to the podosome: Its role in the regulation of podosome assembly

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ARTICLE INFO

Article history:
Received 2 September 2011
Received in revised form
14 December 2011
Accepted 14 December 2011
Available online 4 January 2012

Keywords:
TRPV2
Calcium
Podosome
Macrophage

ABSTRACT

The present study was conducted to investigate localization and function of TRPV2 channel in a mouse macrophage cell line, TtT/M87. We infected an adenovirus vector encoding TRPV2 tagged with c-Myc in the extracellular domain. Immunoreactivity of c-Myc epitope exposed to the cell surface formed a ring structure, which was colocalized with markers of the podosome, namely β-integrin, paxillin and Pyk2. The ring structure was also observed in TRPV2-GFP-expressing cells using total internal reflection fluorescent microscopy. Addition of formyl-Met-Leu-Phe (fMLP) increased the number of podosome and increased the intensity of the TRPV2 signal associated with the podosome. Measurement of subplasmalenmal free calcium concentration ($[Ca^{2+}]_{pm}$) revealed that $[Ca^{2+}]_{pm}$ was elevated around the podosome. fMLP further increased $[Ca^{2+}]_{pm}$ in this region, which was abolished by a TRPV2 inhibitor ruthenium red. Phosphorylated Pyk2 was detected in fMLP-treated cells, and knockdown of TRPV2 reduced the expression of phospho-Pyk2. Introduction of dominant-negative Pyk2 or knockdown of TRPV2 increased the number of podosome. Conversely, elevation of $[Ca^{2+}]_{pm}$ by the addition of ionomycin reduced the number of podosome. These results indicate that TRPV2 is localized abundantly in the podosome and increases $[Ca^{2+}]_{pm}$ by the podosome. The elevation of $[Ca^{2+}]_{pm}$ is critical to regulate assembly of the podosome.

1. Introduction

Calcium regulates various cellular functions and acts as an intracellular messenger [1,2]. Cellular calcium metabolism is regulated tightly, and changes in calcium fluxes occur quite rapidly. Thus, calcium regulates rapid cellular events, for example, contraction of skeletal muscle and exocytosis of neurotransmitters. Calcium is also important for regulating long-term cellular events including contraction of smooth muscle, secretion of hormones, shape change and migration of cells, cell proliferation and gene expression. To regulate long-term effects, calcium entry into the cell is quite important [2,3], and various ligands induce long-term changes in cellular calcium by modulating various types of calcium channels and transporters [4,5].

In macrophages, chemotactic peptide formyl-Met-Leu-Phe (fMLP) activates the calcium messenger system and induces long-term effects such as respiratory burst, shape change and cellular migration. Calcium entry induced by fMLP is critical to induce these long-term effects. We reported recently that fMLP increases calcium entry mainly by acting on the calcium-permeable TRPV2 channel [6]. Most of the TRPV2 locates in an intracellular compartment in unstimulated conditions and, upon stimulation by fMLP, TRPV2 translocates to the plasma membrane by a phosphatidylinositol (PI) 3-kinase-dependent mechanism. Regarding the functional significance of TRPV2, inhibition of fMLP-induced calcium entry by knockdown of TRPV2, blocking translocation of TRPV2 or inhibition of TRPV2 activity markedly reduced fMLP-induced migration [6]. It is thus quite likely that TRPV2 translocated to the plasma membrane regulates cytoskeletal organization and eventually migration of macrophage by modulating calcium entry. The present study was conducted to address the localization and functional role of TRPV2 in the plasma membrane in macrophages. The results indicate that TRPV2 is localized abundantly in the podosome and increases subplasmalenmal free calcium concentration ($[Ca^{2+}]_{pm}$) around the podosome, which is critical for regulation of the podosome assembly.

2. Materials and methods

2.1. Cell culture and transfection

TtT/M87, a mouse macrophage cell line [7], was provided by Dr. Kinji Inoue of Saitama University (Saitama, Japan). These cells were maintained in α-MEM supplemented with 10% fetal calf serum. For production of adenovirus or retrovirus, HEK 293T cells or HEK

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doi:10.1016/j.ceca.2011.12.012

293T-derived platinum-E cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Platinum-E retrovirus packaging cell line was kindly gifted from Toshio Kitamura (University of Tokyo). Mouse peritoneal macrophages were obtained from mouse injected with thioglycollate. Transient transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.).

2.2. Expression vectors

2.2.1. Adenovirus and letrovirus plasmids

Plasmids containing human TRPV2 with an exofacial Myc epitope and EGFP or Strawberry (hTRPV2-Myc-EGFP or -Strawberry) were described previously [6,8]. Briefly, Myc-tag containing EcoRV and BglII restriction enzyme sites was inserted into the first extracellular loop of human TRPV2 by PCR mutagenesis and C-terminal of TRPV2 was fused in-frame with EGFP (Clonthech, Mountain View, CA, U.S.A.). c-Myc-TRPV2-Strawberry or c-Myc-TRPV2-Kate were obtained by exchanging EGFP for strawberry or Kate. Mouse TRPV2-EGFP and TRPV2-Strawberry were similarly constructed. The constructs were subcloned into Gateway entry vector, pENTR3C (Invitrogen). The short hairpin RNA (Sh-RNA) vector was used for RNAi technique.

We made a construct with mouse polymerase III U6 promoter controlling the expression of shRNA, which backward tandemly jointed cytomegalovirus (CMV) promoter controlling EGFP or RFP. The mouse U6 promoter and stuffer sequence, which was between BamH1 and Cla1 restriction enzyme sites, was derived from pSINsi-mU6 vector (Takara, Tokyo, Japan) and CMV-controlled EGFP was derived from C1-EGFP (Invitrogen), and CMV-controlled RFP was exchanged EGFP for RFP. These plasmids were subcloned into pENTR3C (mU6shEGFP/RFP-pENTR). Thus, shRNA expression vectors were constructed by inserting an appropriated synthetic double-stranded oligo nucleotides into the stuffer sequence of the vector. The construct was used for validating the expression and effects of knockdown. The mouse TRPV2 shRNA target sequence of two complementary single-strand oligos GATCCGCATGCTCTGGTAATGATTGCGTGTGCTGTCCGCAATCATTACCAGAGCATGCTTTTTTAT and CGATAAAAAAGCATGCTCTGGTAATGATTCCGGACAGCACACGCAATCATTACCAGAGCATGCG was synthesized (Invitrogen). These oligos were cloned into the BamH1 and Cla1 sites of mU6shEGFP/RFP-pENTR vectors. Adenovirus mouse shTRPV2 RNA vectors (shmTRPV2-EGFP or RFP) were used for transient knockdown. Retrovirus mouse shTRPV2-EGFP or RFP retrovirus vectors were used for stable knockdown of mTRPV2. The stuffer sequence of pSINsi-mU6 vector was used as a negative control (sh-control-EGFP or RFP).

Mouse Akt1 (mAkt1) cDNA and mouse pyk-2 cDNA were obtained from mouse brain total RNA by RT-PCR amplification according to the manufacturer's instruction (Invitrogen). For mAk1 cDNA sequence (GenBank accession number NM_009652), the forward primer 5′-GGGGCCACGGATACCATGAACGA-3′, and the backward primer 5′-CCAGGCCTCAGGCTGTGCCACT-3′ were designed, respectively. The amplification products were resolved by electrophoresis on 1% agarose gel, extracted and subcloned in pCR-Blunt vector (mAkt1-Blunt). Pleckstrin homology domain (PH-mAkt1) was obtained from mAkt1-Blunt vector as template by PCR amplification and was subcloned in pENTE in-frame N-terminal EGFP or TagRFP (PH-mAKT-EGFP/TagRFP).

Same as above procedure, mouse Pyk2 (GenBank accession number NM_172498) cDNA was generated by RT-PCR amplification. The extracted PCR products were subcloned into pCR-Blunt vector. HA-tagged sequence was introduced at the C-terminal of mPyk2 by inverse PCR amplification. C-terminal HA-tagged PRNK (the dominant-negative carboxy terminus Pyk2-related non-kinase) was generated from methionine 685 of the open reading frame of mouse Pyk2 as a start codon. All of these vectors were subcloned into pENTR3C.

Monometric RFP and Strawberry plasmids were provided by Professor Roger Tsien (UCSD). Yellow Cameleon 3.6 pm (YC-pm) was a kind gift from Dr. Atsushi Miyawaki (Riken, Wako, Japan).

Lipofectamine 2000 (Invitrogen) was used for the transient expression or the production of recombinant adenoviruses or rentiviruses. PM-EYFP plasmid was purchased from Clonthech. All of the constructs were confirmed by ABI 3100 DNA Sequenser.

2.2.2. Construction of recombinant adenoviruses

Various adenovirus vectors were obtained by recombination of pENTR3C vectors, which contained above described genes, into pAd/CMV/V5/DEST vector according to the manufacturer's protocols (Invitrogen). The results constructs were transfected into HEK293 cells, recombinant adenoviruses were serially amplified and purified.

2.2.3. Establishment of stable transfectants

Retrovirus vectors were prepared by using p-LEGFP-N1 retroviral vector (Clonthech), which was converted into a Gateway destination vector. The vectors were introduced into platium-E retrovirus packaging cell line. After 24–48 h, the medium was harvested and filtered through a 0.22 μm filter (Toyobo, Tokyo, Japan). Retrovirus-containing medium was supplemented with polybrene (10 μg/ml) and transferred to subconfluent, proliferating TtT/M87 cells. A subclone of the TtT/M87 cell line stably expressing, respectively, retrovirus constructs was obtained with G418 selection.

2.3. Imaging experiments

2.3.1. Total internal reflection fluorescence microscopy

TtT/M87 cells incubated in Hank's balanced salt solution (Nissui, Tokyo, Japan) were subjected to imaging. Fluorescence images were captured using an inverted microscope (IX-71, Olympus, Tokyo, Japan) equipped with a 100× total internal reflection fluorescent microscopy (TIRFM) objective (N.A.1.45, Olympus) and a cooled CCD camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan). Briefly, a 488 nm argon-ion laser brought through the right illumination port of an Olympus IX71 fluorescence microscope. The laser light reflected from a dichroic mirror passes through a high-numerical aperture objective lens and totally internally reflected by the glass–water interface ($n = 1.37$) was obtained by using a micrometer. Images were acquired using an ORCA-ER camera (Hamamatsu Photonics) driven by Aqua Cosmos imaging software (Hamamatsu Photonics).

2.3.2. Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) images were captured using an inverted microscope (IX-81, Olympus, Tokyo, Japan) equipped with a cooled 3CCD camera (ORCA-3CCD, Hamamatsu Photonics). The excitation light source was provided by a 150 W Xenon lamp (Olympus) for epifluorescence imaging. The Mirror Cassette XF 88-2 (Olympus) was used for simultaneous capturing images of CYP and Venous. The cells expressing YC3.6pm were incubated in Hank's balanced salt solution. Live cell images were acquired at a rate of 60 s, and stimulation by a point source of the chemoattractant from a micropipette containing 10 μM fMLP was performed.

2.3.3. Immunofluorescence microscopy

For immunofluoro-microscopic analysis, cells were grown on glass coverslips. Cells were fixed for 30 min at room temperature in 4% paraformaldehyde PBS solution and washed with PBS. For staining of extracellular myc-tagged hTRPV2, cells were not permeabilized first and blocking of non-specific binding was performed