Cell Calcium 54 (2013) 307–319

Contents lists available at ScienceDirect

Cell Calcium

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

Activation of TRPC4β by $G\alpha_i$ subunit increases Ca^{2+} selectivity and controls neurite morphogenesis in cultured hippocampal neuron

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ARTICLE INFO

Article history:
Received 29 November 2012
Received in revised form 27 July 2013
Accepted 29 July 2013
Available online 14 August 2013

Keywords:
TRPC
Transient receptor potential channel
TRPC4
Galphai2
Gi2
Dendrite
Neurite
Calcium

ABSTRACT

The ubiquitous transient receptor potential canonical (TRPC) channels function as non-selective, Ca^{2+}-permeable channels. TRPC channels are activated by stimulation of $G\alpha_q$-PLC-coupled receptors. Here, we report that TRPC4/TRPC5 can be activated by $G\alpha_i$. We studied the essential role of $G\alpha_i$ subunits in TRPC4 activation and investigated changes in ion selectivity and pore dilation of the TRPC4 channel elicited by the $G\alpha_{i2}$ subunit. Activation of TRPC4 by $G\alpha_{i2}$ increased Ca^{2+} permeability and Ca^{2+} influx through TRPC4 channels. Co-expression of the muscarinic receptor (M2) and TRPC4 in HEK293 cells induced TRPC4-mediated Ca^{2+} influx. Moreover, both TRPC4β and the TRPC4β-$G\alpha_{i2}$ signaling complex induced inhibition of neurite growth and arborization in cultured hippocampal neurons. Cells treated with KN-93, a CaMKII inhibitor, prevented TRPC4- and TRPC4-$G\alpha_{i2}{}^{Q205L}$-mediated inhibition of neurite branching and growth. These findings indicate an essential role of $G\alpha_i$ proteins in TRPC4 activation and extend our knowledge of the functional role of TRPC4 in hippocampal neurons.

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1. Introduction

Transient receptor potential canonical (TRPC) channels are receptor-operated Ca^{2+}-permeable cation channels that are involved in many physiological processes [1,2]. TRPC channels can be activated by G-protein-coupled receptor (GPCR)-$G\alpha_q$-PLC pathway. The activation mechanisms of TRPC4 and TRPC5 have been proposed G-protein-coupled receptors of the $G\alpha_{q/11}$-PLCβ and receptor tyrosine kinases-PLCγ [3,4]. However, the exact mechanism by which the channels are activated in this pathway remains largely unknown [3–5]. Diacylglycerol (DAG) cannot activate TRPC4 channels but are facilitated by internal and external Ca^{2+} [3].

Recently, we showed that TRPC4 and TRPC5 are activated primarily by selective $G\alpha_i$ subunits rather than by $G\alpha_q$ [6,7]. TRPC4 is activated by several $G\alpha_i$ subunits, most prominently by $G\alpha_{i2}$, while TRPC5 is activated by $G\alpha_{i3}$. Activation of $G\alpha_i$ by muscarinic M2 receptors or expression of constitutively active $G\alpha_i$ mutants equally and fully activates TRPC4 and TRPC5 channels. Moreover, we have shown that both TRPC4 and TRPC5 are activated by direct interaction of their conserved C-terminal SEC14-like and spectrin-type domains (SESTD) with $G\alpha_i$ subunits. However, the effect of interaction with selective $G\alpha_i$ subunits on TRPC channel function and the functional consequences of this activation mechanism remain unknown.

Traditionally, ionic selectivity and conductance are considered fundamental properties of ion channels. However, several studies have shown that the pore properties of ion channels can be changed by agonist stimulation or protein interaction. Among TRP

Abbreviations: TRPC, transient receptor potential canonical; GPCR, G-protein-coupled receptor; EGFP, enhanced GFP; GTPγS, guanosine5′-3-O-(thio)triphosphate; PTX, pertussis toxin; PIP_2, phosphatidylinositol 4,5-bisphosphate; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; PLC, phospholipase C; GI, gastrointestinal; n.s., not significant.

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http://dx.doi.org/10.1016/j.ceca.2013.07.006

channels, TRPV1 and TRPA1 were shown to undergo pore dilation and selectivity changes subsequent to activation by ligand stimulation [8,9]. Another very important example is the ORAI1 channel, for which activation by STIM1 resulted in high Ca^{2+} selectivity, low Cs^{+} permeability and a narrow pore [10]. These results suggest the possibility that activation of TRPC4 through interaction with $G\alpha_i$ subunits may cause changes in the pore properties of TRPC4 and consequently affect cellular functions.

TRPC4 is abundantly expressed in smooth muscle and in the mammalian brain [11]. In visceral smooth muscle cells, the physiological role of TRPC4 is one of molecular identity with TRPC6; both are non-selective cation (NSC) channels activated by muscarinic receptor stimulation (mI_{CAT}) [12]. The signaling pathway that activates mI_{CAT} is well explained by our model in that TRPC4 is activated by M2 muscarinic stimulation. In brain, TRPC4 is highly expressed in the frontal cortex, the pyramidal cell layer of the hippocampus, and the dentate gyrus [13]. Although TRPC4 has been implicated in neurite extension in post-mitotic neurons [14], the functional role of TRPC4 and the TRPC4-$G\alpha_i$ signaling complex in brain are yet to be elucidated.

We report here that interaction with $G\alpha_i$ subunits not only activates TRPC4β but also changes its channel properties, increasing its Ca^{2+} permeability. Moreover, we present evidence that TRPC4β controls dendrite morphogenesis in cultured hippocampal neurons and show that this function is augmented when TRPC4β is activated by $G\alpha_i$. Together, our results demonstrate that dynamic pore behavior can be regulated by intracellular protein interaction and reveal a novel functional role of TRPC4β and the TRPC4β-$G\alpha_i$ signaling complex in mammalian brain.

2. Materials and methods

2.1. Cell culture and transient transfection; cDNA clones

Human embryonic kidney (HEK293) cells (ATCC, Manassas, VA) were maintained according to the supplier's recommendations. For electrophysiological recording, cells were seeded in 12-well plates. The following day, the cells were transfected with 0.5 μg/well of pEGFP-N1 vector containing mouse TRPC4β-EGFP cDNA using the transfection reagent FuGENE 6 (Roche Molecular Biochemicals) as detailed in the manufacturer's protocol. Co-expression of the TRPC4 channel with G-proteins was achieved through a channel to G-protein transfection ratio of 1:1. After 30–40 h, the cells were trypsinized and transferred to a small recording chamber (RC-11, Warner Instruments) for whole-cell recording. Human $G\alpha_{i1}^{G202T}$, $G\alpha_{i2}^{G203T}$, $G\alpha_{i3}^{G202T}$ (Missouri S&T cDNA Resource Center) and human $G\alpha_{i2}^{Q205L}$ were cloned into pcDNA3.1+. Human TRPC4α was cloned into pcDNA3 (given kindly by Dr. Shuji Kaneko). The mutation of human $G\alpha_{i2}^{Q205L/G2A/C3S}$ and mouse TRPC4βDN (571-LFW-573/AAA) was created using the QuickChange site-directed mutagenesis kit (Stratagene). For immunostaining, HEK293 cells were transfected with mouse TRPC4β, $G\alpha_{i2}^{Q205L}$, and $G\alpha_{i2}^{Q205L/G2A/C3S}$ using lipofectamine 2000, and cells were observed after 24 h.

2.2. Western blotting and co-immunoprecipitation

Transfected cells were collected and lysed in 300 μl of binding buffer (50 mM HEPES, pH 7.4, 120 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$, complete protease inhibitor mixture tablet, phosphatase inhibitor cocktail tablet (Roche Applied Science) and 0.5% Triton X-100). The lysates were sonicated, and insoluble material was removed by centrifugation at 13,300 × *g* for 10 min. For co-immunoprecipitation of TRPC4β-GFP with $G\alpha_{i2}^{Q205L}$ and $G\alpha_{i2}^{Q205L/G2A/C3S}$ and TRPC4α-GFP with $G\alpha_{i2}^{Q205L}$, anti-GFP antibody (1 μg, Invitrogen, A11122) was added to 100 μl cell extract and the mixture was incubated for 12 h at 4 °C. Then, 50 μl of a 1:1 slurry of protein G-Sepharose 4B beads was added to the antibody-extract mix and incubated for 12 h at 4 °C. After washing the beads three times with binding buffer; proteins were released from the beads with 50 μl of 2× SDS-loading buffer and analyzed by 10% or 8% SDS-PAGE. $G\alpha_{i2}$ was co-immunoprecipitated with GFP antibody and probed with mouse monoclonal anti-$G\alpha_{i2}$ antibody (2 μg, Santa Cruz, sc-13534) [7].

2.3. Neuronal cultures, transfection, and immunocytochemistry

Dissociated hippocampal neurons were cultured as previously described with some modifications [15]. Briefly, isolated hippocampus from BL/6 P0 mouse pups were triturated in Neurobasal medium (GIBCO) supplemented with DNAse (20 μg/ml) and 10% FBS after trypsinizing for 15 min. Cells were plated at 5×10^4 per well in 500 μl of Neurobasal medium supplemented with B27 (GIBCO) and Glutamax (GIBCO) on poly-D-lysine (Sigma)-coated 12-mm coverslips in 24-well culture plates. Another 500 μl of medium with arabinofuranosyl cytidine (10 mM) was added, and 20–30% of the medium was exchanged every other day until the cells were used in the experiments. At 6 or 7 DIV, neurons were transfected using lipofectamine 2000 and then maintained for 6–7 days. Briefly, DNA or siRNA (up to 1 μg/well, control/TRPC4 siRNA; Santa Cruz, sc-37007/sc-42669) were mixed with lipofectamine 2000. The DNA mixture was incubated for 20 min in the dark and then added to the neurons in opti-MEM at 37 °C in 5% CO_2 for 30 min. For immunostaining, HEK293 cell lines and neurons were fixed in 4% paraformaldehyde (PFA) in PBS buffer for 15 min at room temperature (RT). After fixation, neurons were washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked for 1 h at RT with PBS containing 1% BSA (Sigma–Aldrich). After co-transfection of HEK293 cells with TRPC4-GFP and $G\alpha_{i2}^{Q205L}$, the cells were immunostained using mouse anti-G_{i2} antibody (Santa Cruz; 1:100) for 1 h at RT and Alexa Fluor Texas Red-conjugated goat anti-mouse antibody (1:300) for 1 h at RT. After secondary antibody incubation, the cells were washed with PBS three times for 5 min, and coverslips were mounted using Elvanol mounting medium.

2.4. Imaging, microscopy, and quantification

Images were acquired by using an Olympus FV-1000 confocal microscope driven by FluoView 1000 with a 60× 1.35 NA oil lens for HEK393 cell lines. Cells were excited with 488 nm (from an argon laser) and 559 nm light (from a diode laser). Images for transfected neurons were acquired on a Nikon Eclipse Ti microscope (Japan) and cells were excited with 495 nm (epifluorescence-mercury lamp). Transfected neurons were analyzed by two analysis methods (Sholl and total neurite length). For Sholl analysis, concentric circles with 10 μm differences in diameter were drawn around the cell body, and the number of neurites (dendrites) crossing each circle was manually counted using NIS software. To measure total neurite length, the NeuronJ plugin was used in conjunction with ImageJ. Data in the graphs are represented as averages plus SEMs from three or five batches of neuron.

2.5. Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)

HEK293 cells were loaded with the Ca^{2+}-sensitive fluorescent indicator fura-2-AM (5 μM, 30 min) in normal Tyrode's solution at room temperature, and the unloaded indicators were washed out with fresh solution. Recording of $[Ca^{2+}]_c$ was performed with a cooled CCD camera (CoolSNAP *HQ*^{2}, Photometrics, USA) attached to the Nikon Eclipse Ti inverted microscope to measure

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