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Endogenous TRPV1 stimulation leads to the activation of the inositol phospholipid pathway necessary for sustained Ca^{2+} oscillations

László Pecze *, Walter Blum, Thomas Henzi, Beat Schwaller

Anatomy, Department of Medicine, University of Fribourg, Route Albert-Gockel 1, Fribourg, CH-1700, Switzerland

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ABSTRACT

Sensory neuron subpopulations as well as breast and prostate cancer cells express functional transient receptor potential vanilloid type 1 (TRPV1) ion channels; however little is known how TRPV1 activation leads to biological responses. Agonist-induced activation of TRPV1 resulted in specific spatiotemporal patterns of cytoplasmic Ca^{2+} signals in breast and prostate cancer-derived cells. Capsaicin (CAPS; 50 μM) evoked intracellular Ca^{2+} oscillations and/or intercellular Ca^{2+} waves in all cell lines. As evidenced in prostate cancer Du 145 cells, oscillations were largely dependent on the expression of functional TRPV1 channels in the plasma membrane, phospholipase C activation and on the presence of extracellular Ca^{2+} ions. Concomitant oscillations of the mitochondrial matrix Ca^{2+} concentration resulted in mitochondria energization evidenced by increased ATP production. CAPS-induced Ca^{2+} oscillations also occurred in a subset of sensory neurons, yet already at lower CAPS concentrations (1 μM). Stimulation of ectopically expressed TRPV1 channels in CAPS-insensitive NIH-3T3 cells didn't provoke CAPS-triggered Ca^{2+} oscillations; rather it resulted in low-magnitude, long-lasting elevations of the cytosolic Ca^{2+} concentration. This indicates that sole TRPV1 activation is not sufficient to generate Ca^{2+} oscillations. Instead the initial TRPV1-mediated signal leads to the activation of the inositol phospholipid pathway. This in turn suffices to generate a biologically relevant frequency-modulated Ca^{2+} signal.

1. Introduction

Calcium ions (Ca^{2+}), universal signaling molecules, are widely recognized to play a fundamental role in the regulation of various biological processes. Many cytosolic and mitochondrial activities are driven in a Ca^{2+}-dependent manner. Therefore, each cell possesses sophisticated mechanisms for the precise regulation of cytoplasmic (c_{cyt}), endoplasmic reticulum luminal (c_{ER}) and mitochondrial matrix (c_{mito}) Ca^{2+} concentrations. Since Ca^{2+} regulates the cell cycle at several stages, Ca^{2+} signaling is importantly involved in cell-fate determination (quiescent state, proliferation or cell death). Mitogenic compounds such as platelet-derived growth factor, vasopressin, prostaglandin, bombesin or EGF evoke repetitive Ca^{2+} transients and also induce inositol trisphosphate ($InsP_3$) production [1,2]. In Swiss 3T3 cells, increases in c_{cyt} evoked by mitogenic compounds are essential, but not sufficient to induce DNA synthesis and proliferation [3]. Moreover the frequency of base-line spiking Ca^{2+} oscillations in cultured human embryonic kidney (HEK) cells is directly related to cell proliferation [4]. In postmitotic neurons, Ca^{2+} oscillations regulate a variety of neuronal processes including excitability, associativity, neurotransmitter release, synaptic plasticity and gene transcription [5].

The signaling process leading to Ca^{2+} oscillations is composed of the following steps: I) A ligand binds to its receptor coupled to heterotrimeric G proteins, II) the $G_{\alpha q}$ subunit of a heterotrimeric G protein dissociates from the G protein-receptor complex and activates phospholipase C, III) phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate ($InsP_3$), which then releases Ca^{2+} from intracellular stores via binding to the inositol trisphosphate receptor ($InsP_3R$) [6] and diacylglycerol (DAG), which activates protein kinase C (PKC) and IV) activation of $InsP_3R$ by $InsP_3$ leads to the quantal release of Ca^{2+} ions from the endoplasmic reticulum [7]. The shape of the Ca^{2+} signal is correlated with the $InsP_3$ concentration, i.e. the stimulation intensity: weak activation results in single Ca^{2+} spikes, increasing the stimulation leads to slow base-line spiking oscillations, followed by fast base-line spiking oscillations, sinusoidal Ca^{2+} oscillations and finally non-oscillating signal-plateau Ca^{2+} responses [8]. Of note, Ca^{2+} oscillations occur within a certain range of agonist stimulation. Ryanodine receptors have structural and functional similarity to $InsP_3R$, but show no sensitivity to $InsP_3$ [9]. One of the functions of ryanodine receptors is to amplify the $InsP_3$-mediated release of Ca^{2+} [10]. The unique bell-shaped dependence of $InsP_3R$ and ryanodine receptors on c_{cyt} allow for the repetitive release of Ca^{2+} ions from the endoplasmic reticulum [11]. This is what is observable as Ca^{2+} oscillations.

* Corresponding author at: Anatomy, Department of Medicine, University of Fribourg, Route Albert-Gockel 1, CH-1700 Fribourg, Switzerland.
E-mail address: laszlo.pecze@unifr.ch (L. Pecze).

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The transient receptor potential cation channel subfamily V member 1 (TRPV1) triggers intracellular signaling mechanisms by an increase of c_{cyt}, when it is activated by multiple pain-inducing stimuli including heat, acids and pungent compounds [12]. TRPV1 is activated by selective potent natural agonists such as capsaicin (CAPS) and resiniferatoxin (RTX), pungent compounds found in chili pepper and in a tropical plant called *Euphorbia resinifera*, respectively [13]. Upregulation of TRPV1 channels in neoplastic breast and prostate tissue compared to normal tissue has been reported before [14,15], but little is known about the channel's physiological function and the likely pathological consequences in these neoplasms. Virtually all pharmacological and molecular methods used to examine the function of these channels resulted in a decrease of cell viability. These methods included molecular up- or downregulation of the channels and activation or inhibition of channels with natural exogenous agonists or synthetic antagonists [16,17]. The activation of these channels leads to an increase in c_{cyt} in breast and prostate cell lines, but the previously published studies presented only the average of evoked Ca^{2+} cytoplasmic signals within the entire cell populations [17–19]. This method blurs the spatiotemporal character of individual intracellular Ca^{2+} signals, which is essential to understand how TRPV1-mediated stimuli influence the cell behavior at the single cell level.

In this study, spatiotemporal recordings of c_{cyt} and c_{mito} were collected and analyzed; moreover selected mitochondrial functions (membrane potential, ATP production) were analyzed as well. The results revealed the connection between TRPV1 channels and the inositol phospholipid pathway and moreover how TRPV1-mediated Ca^{2+} signals are processed to biologically relevant frequency-modulated Ca^{2+} oscillations. It is known that TRPV1 channels are modulated by phospholipids such as PIP2 [20,21], but our results revealed that the activation of endogenous TRPV1 leads to the production of inositol trisphosphate, thereby reducing the levels of PIP2 in the plasma membrane creating a negative feedback loop.

2. Materials and Methods

2.1. Reagents

Capsaicin (CAPS), a TRPV1 agonist and capsazepine (CapZ), a well-characterized antagonist of TRPV1 were dissolved in DMSO at a concentration of 100 mM (all from Sigma-Aldrich, St. Louis, MO). Resiniferatoxin (RTX) from the LC Laboratories (Woburn, MA) was dissolved in ethanol at a concentration of 2 mM. 13(S)-Hydroxyoctadeca-9Z,11E–dienoic acid (13(S)-HODE), an endogenous agonist of TRPV1 was obtained from Sigma-Aldrich. Suramin from Adipogen (Liestal, Switzerland) was dissolved in double distilled water. Phospholipase C inhibitor U-73122 were from Tocris (Ellisville, MO). The compounds were further diluted with buffer solution used for Ca^{2+}-imaging experiments that contained (in mM): NaCl 138, Na_2PO_4 8, $CaCl_2$ 2, $MgCl_2$ 0.5, KCl 2.7, KH_2PO_4 1.6; pH 7.4. The final concentration of the solvents were <0.1% in all experimental solutions. At these concentrations the solvents did not affect/modify the evoked Ca^{2+} responses in control experiments (data not shown). Ethylene glycol tetra acetic acid (EGTA) was dissolved with NaOH in double distilled water at basic pH (pH > 8.0) and then the pH was adjusted to 7.4 with HCl. The nuclear stain Hoechst 33,342, the mitochondrial marker MitoTracker Red CMXRos and the plasma membrane marker CellMask™-Orange were purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

2.2. Plasmids and cell lines

The cDNA of the human TRPV1 channel (hTRPV1) was amplified from RNA isolated from human trigeminal ganglion tissue as previously described [22]. The pGFP-TRPV1 plasmid resulting in a GFP-TRPV1 fusion protein, as well as the plasmid pTRPV1 encoding full-length TRPV1 were used in this study. The plasmid encoding $InsP_3$ 5-phosphatase (pIRES-InsP3–5P-GFP) was a kind gift from Christophe Erneux, IRIBHM, Bruxelles [23]. The BFP-KDEL plasmid for ER visualization was a gift from Gia Voeltz; Addgene plasmid #49,150. The mCherry-hCdt1 plasmid was a kind gift of Prof. H. Miyoshi (Riken, Japan). This plasmid was used to label nuclei in red color. The control plasmid pEGFP-C1 coding for EGFP was from Clontech (Palo Alto, CA). In order to generate lentivirus encoding the red Ca^{2+} indicator CAR-GECO1 (the CMV-CAR-GECO1 plasmid was a gift from Robert Campbell; Addgene plasmid #45,493), a fragment encoding CAR-GECO1 was cloned into lentiviral expression vector pLVTHM (pLVTHM was a gift from Didier Trono; Addgene plasmid # 12,247)). The GFP cassette in pLVTHM was replaced with cDNAs coding for the respective Ca^{2+} indicator proteins. Briefly, the required cDNA fragment coding for full-length CAR-GECO1 was synthesized by PCR using the primers FW_PmeI_Car-Geco1 (5′- CTT TGT TTA AAC ATG GTC GAC TCA TCA CGT-3′) and RV_*Nde*I_Car-Geco1 (5′-ATT CCA TAT GCT ACT TCG CTG TCA TCA T-3′). The amplicon was digested with *PmeI* and *NdeI* and inserted into the unique sites of the pLVTHM vector to produce the final pLV-CAR-GECO1 plasmid. The lentivirus was produced by the calcium phosphate transfection method using HEK 293 cells and three plasmids: one of the expression plasmids (e.g. pLV-CAR-GECO1 or pLV-mito-CAR-GECO1), the envelope plasmid (pMD2G–VSVG Addgene plasmid #12,259) and the packaging plasmid (psPAX2, Addgene plasmid #12,260). Viral containing supernatants were collected after 48 h and 72 h, filtered, aliquoted and frozen at −80 °C, as described before [24]. Human prostate (PC-3, LNCaP, Du 145) and breast (MCF7, BT-474, MDA-MB-231) cancer cell lines and HEK 293 cells of human embryonic kidney origin were purchased from ATCC (Manassas, VA, USA). Non-transfected NIH-3T3 and rat TRPV1-expressing NIH-3T3 murine fibroblast cells (NIH-3T3^{rTRPV1}) were a kind gift from Dr. Zoltan Olah, University of Miskolc, Hungary. In NIH-3T3^{rTRPV1} cells, the metallothionein promoter is used to drive the expression of full-length rat TRPV1 with a short 12 amino acid ε-tag [25]. Cells were cultivated in DMEM containing 10% fetal calf serum and antibiotics (penicillin and streptomycin) at 37 °C/5% CO_2. DRG primary cultures were prepared from E15 rat embryos as previously described [22]. DRG cell cultures were maintained in DMEM containing 5% horse serum and 100 ng/ml nerve growth factor (Sigma-Aldrich) to promote neuronal survival and differentiation. After 2 days *in vitro* primary DRG cultures were used for the experiments. Du 145 cells stably expressing the Ca^{2+} indicator protein CAR-GECO1 was generated with lentiviral infection. In some experiments, Du 145, MCF7, HEK 293 and NIH-3T3 cells were transiently transfected using the TransIT-2020 transfection reagent according to manufacturer's instructions (Myrus, Madison, WI).

2.3. Immunofluorescence staining

Cells were prepared for immunofluorescence analysis as described previously [26]. As a positive control, MCF7 cells were transiently transfected with plasmids encoding human TRPV1 cDNA. The following antibodies were used for staining: anti-TRPV1 (1:500; rabbit polyclonal, Alomone Labs #ACC-030), Cell nuclei were stained with 5 μg/ml DAPI (Invitrogen) in Tris-buffered saline for 10 min, and mounted with Hydromount solution (National Diagnostics, Atlanta, GA). Images were acquired with a confocal microscope DMI6000 integrated to a Leica TCS-SP5 workstation (Leica, Wetzlar, Germany).

2.4. Ca^{2+} imaging

Cells grown on collagen-coated glass bottom 35 mm dishes (MatTek Corp., Ashland, MA) were loaded with the cell permeable acetoxymethyl (AM)-ester form of the indicator dyes. The following dyes were used: for the cytoplasmic free Ca^{2+} concentration (c_{cyt}): Fluo-4-AM (1 μM; Life Technologies, Grand Island, NY) and for the mitochondrial free Ca^{2+} concentration (c_{mito}): Rhod-2-AM (1 μM; Life Technologies) diluted in cell culture media for 20 min at room temperature. After loading cells