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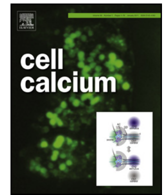
YCECA-1715; No. of Pages 11
ARTICLE IN PRESS
Cell Calcium xxx (2015) xxx–xxx

Contents lists available at ScienceDirect
Cell Calcium
journal homepage: www.elsevier.com/locate/ceca



TRPV3 channels mediate Ca^{2+} influx induced by 2-APB in mouse eggs

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A R T I C L E I N F O

Article history:
Received 28 July 2015
Received in revised form 9 December 2015
Accepted 11 December 2015
Available online xxx

Keywords:
TRPV3 channel
2-APB
Ca^{2+} influx
Actin cytoskeleton
IP_3R1 receptor

A B S T R A C T

Fertilization in mammals is initiated when a sperm fuses with a mature MII oocyte, also known as egg, and triggers a plethora of finely controlled processes identified as egg activation. The completion of all events of egg activation is driven by and depends on a series of repetitive calcium (Ca^{2+}) increases (Ca^{2+} oscillations), which rely on Ca^{2+} influx from the extracellular media. Ca^{2+} channels on the egg plasma membrane (PM) are thought to mediate this influx. The TRP Ca^{2+} channel TRPV3 is differentially expressed during oocyte maturation, being most active at the MII stage. Specific stimulation of TRPV3 channels promotes Ca^{2+} influx sufficient to induce egg activation and parthenogenesis. Here, we explore the function and distribution dynamics of the TRPV3 channel protein during maturation. Using dsRNA, *TrpV3* overexpression, and inhibitors of protein synthesis, we modified the expression levels of the channel and showed that the TRPV3 protein is synthesized and translocated to the PM during maturation. We demonstrated that 2-APB at the concentrations used here to promote Ca^{2+} influx in eggs, specifically and reversibly targets TRPV3 channels without blocking IP_3R1. Finally, we found that the activity of TRPV3 channels is dependent upon an intact actin cytoskeleton, suggesting an actin-based regulation of its expression and/or function on the PM. Collectively, our results show TRPV3 is a target of 2-APB in eggs, a condition that can be used to induce parthenogenesis. The need of an intact actin cytoskeleton for the function of TRPV3 channels in oocytes is a novel finding and suggests the rearrangements of actin that occur during maturation could regulate both the presence on the PM and/or the function of TRPV3 and of other Ca^{2+} channels involved in oocyte maturation and fertilization.

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1. Introduction

Egg activation is the process that transforms a ready-to-be-fertilized MII oocyte, also known as an egg, into a one-cell embryo, the zygote, capable of initiating development to term. Egg activation starts with the fertilization induced changes in the intracellular concentration of calcium ($[Ca^{2+}]_i$), $[Ca^{2+}]_i$ oscillations, which in turn trigger all events of activation including cortical granule exocytosis and prevention of polyspermy, completion of meiosis with subsequent formation of the male and the female haploid pronuclei (PN) and the first mitotic cleavage (reviewed in [1]). $[Ca^{2+}]_i$ increases are the universal signal for egg activation events [2], and Ca^{2+} influx is necessary to assure complete and successful activation in mammalian [3–5] and in arthropods eggs (e.g. *Drosophila* [6]). In mammals, Ca^{2+} influx also occurs prior to fertilization during oocyte maturation, which refers to the transition from prophase I to the metaphase II stage of meiosis (MII stage) that fully-grown oocytes undergo in response to a surge in Luteinizing hormone (LH) levels. The MII oocyte stage is the mature egg that is ovulated and subsequently fertilized in mammals [7–9]. The Ca^{2+} influx that occurs during maturation underlies the spontaneous oscillations

Abbreviations: TRPV3, transient receptor potential, subfamily Vanilloid, member 3; 2-APB, 2-aminoethoxydiphenyl borate; MII, metaphase II of the second meiosis; IP_3R, inositol 1,4,5-trisphosphate receptor; PM, plasma membrane; SERCA, Sarco/endoplasmic reticulum Ca^{2+}-ATPase; STIM, stromal interaction molecule; SOCE, store-operated Ca^{2+} entry; CRAC, calcium release-activated calcium; GV, germinal vesicle; PN, pronucleus; PLCζ, phospholipase zeta; MPF, maturation-promoting factor; RFP, ruby fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; LatA, latrunculin A.

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http://dx.doi.org/10.1016/j.ceca.2015.12.001
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observed in prophase-arrested oocytes [10,11], also known as germinal vesicle (GV) oocytes, as well as the progressive increase in the Ca^{2+} content of the endoplasmic reticulum (ER), the main Ca^{2+} store of oocytes and eggs, which occurs in preparation for fertilization [12,13]. Despite the importance of Ca^{2+} influx for mammalian oocytes and eggs, the channel(s) that mediates it, is presently unknown.

External Ca^{2+} can enter cells through channels and/or transporters. The T-type voltage-gated Ca^{2+} channels (CaV 3.2; [14]) were one of the first characterized channels in mouse eggs [15], although their function remains elusive, as CaV 3.2-deficient female mice are fertile [16]. Besides, the changes in membrane potential during mammalian fertilization are negligible [17] and the resting potential of mouse eggs, which is close to the activation threshold of CaV channels, ensures the great majority of these channels are inactive before and after fertilization. A voltage-independent member of the family of TRP channels, TRPV3, was also reported to be active in mouse eggs [18]. High TRPV3 expression was first found in brain, spinal cord, testis, skin, oral cavity and gastrointestinal tract ([19–21] reviewed in [22]). Native TRPV3 currents were first reported in keratinocytes where exposure to 100 μM 2-APB, a known activator of TRPV3 channels, elicited changes in whole cell currents in cells previously sensitized by temperature [23]. Remarkably, endogenous TRPV3 channels in MI oocytes and eggs respond to 2-APB treatment without temperature sensitization, suggesting different regulation or sensitization of the channels in oocytes and eggs [18]. In this study, we expressed the TRPV3 channel tagged with a fluorescent protein and closely follow its localization during maturation. Accordingly, we showed the exogenous channel protein is synthesized during maturation and is translocated to the PM where it becomes fully functional at the MII stage.

2-APB was initially characterized as a specific inhibitor of IP_3R1 [24], an ubiquitously expressed channel that mediates intracellular Ca^{2+} release in most cell types [25]. However, later on, 2-APB was shown to block SERCA [26] and other channels [27] including TRP channels and it was, therefore, defined as an "unspecific" blocker [28]. Further, 2-APB was shown to activate rather than inhibit members of the TRPV sub-family of TRP channels, TRPV1, TRPV2 and TRPV3 [29], and in fact, it is the most used activator of TRPV3 channels [23,29,30]. A more recent addition to the channels sensitive to the stimulatory action of 2-APB is ORAI3 [31], one of the proteins that are molecular correlates, together with STIM, of SOCE or CRAC channels [27]. Accordingly, 2-APB was used to test the presence of ORAI3 in mouse oocytes and eggs, and the Ca^{2+} influx promoted by addition of 2-APB was interpreted as demonstration of ORAI3 expression in oocytes and eggs [32]. However, this result is in conflict with the demonstration that 2-APB is unable to induce Ca^{2+} influx in *TrpV3*-KO eggs (at 100 μM [18]). Using molecular and functional assays we addressed this discrepancy here and show the agonist effect of 2-APB on Ca^{2+} influx in mouse oocytes and eggs is exclusively mediated by TRPV3 [11].

Actin is a major cytoskeleton protein found ubiquitously expressed in eukaryotic cells. Actin-based cytoskeleton complexes are involved in the dynamic transport of channels to the PM including endocytosis/exocytosis and intracellular trafficking [33]; it has also been reported to modulate the activity of channels on the PM [34]. Cytoplasmic actin filaments possess multiple other functions, including pivotal roles in mitosis and meiosis [35]. In the later, actin microfilaments are related with positioning and assembly of the meiotic spindle as well as with the general organization and redistribution of organelles [8,35]. To that end, actin interacts with the Arp2/3 protein complex that becomes activated in the cortical region, where it nucleates a thick cortical actin layer termed "actin cap", which is essential for cytoplasmic flow [36]. This cortical reorganization also correlates with changes in the functional expression of TRPV3 channels [18], suggesting a close relationship between actin reorganization, redistribution, and incorporation of these channels onto the PM. In this study in mouse oocytes and eggs, we hypothesized that the distribution and/or activity of TRPV3 channels may be regulated by the organization of actin microfilaments. Our results suggest that an intact actin cytoskeleton is required for the functional presence of TRPV3 channels on the PM, raising the possibility that microfilaments may regulate its function as well as that of other PM channels involved in Ca^{2+} homeostasis in oocytes and eggs.

2. Materials and methods

2.1. Collection of mouse oocytes

GV oocytes were collected from the ovaries of 6- to 10-week-old CD-1 female mice 44–46 h after injection of 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma). Cumulus intact GV oocytes were recovered in a HEPES–buffered Tyrode's lactate solution (TL-HEPES) containing 5% heat-treated fetal calf serum (FCS; Gibco) and 100 μM IBMX. Cumulus cells were removed by repetitive pipetting and denuded oocytes were matured in vitro for 12–14 h in IBMX-free Chatot, Ziomek, and Bavister (CZB) [37] media supplemented with 3 mg/ml bovine serum albumin (BSA) or 0.1% polyvinyl alcohol (PVA; Sigma, St Louis, MO) under mineral oil at 37 °C in a humidified atmosphere of 5% CO_2. MII oocytes were collected from the oviducts 12–14 h after administration of 5 IU of human chorionic gonadotropin (hCG), which was administered 46–48 h after PMSG. Cumulus cells were removed with 0.1% bovine testes hyaluronidase (Sigma). All animal procedures were performed according to research animal protocols approved by the University of Massachusetts Institutional Animal Care and Use Committee.

2.2. Plasmid and dsRNA preparation

Mouse *TrpV3* (m*TrpV3* cDNA) was kindly provided by Dr. Clapham Lab (Boston Children's Hospital-Harvard medical school). *TrpV3* was subcloned into a pcDNA6B/his-myc B vector between the *Xho*I and *Age*I restriction sites. To determine the subcellular distribution, *TrpV3* was N-terminal tagged with Ruby, RFP-TRPV3. Double stranded RNA (dsRNA) oligonucleotides against *TrpV3* genes were synthesized by the MEGAclear Kit (or Cell to cDNA Kit). DNA fragments of approximately 520 bp in length containing coding sequences for the proteins to be "knock down" were amplified by using PCR. Each primer used in the PCR contained a 5′ T7 RNA polymerase binding site (TAA TAC GAC TCA CTA TAG GG) followed by sequences specific for *TrpV3*. Primer sequence used to generate specific dsRNA was obtained as follows: *TrpV3*, GenBank accession no. NM145099, sense-primer: 5′-CATCA CCCTG ACCCT TGTCT; antisense-primer: 5′-GCTGA AGCTG CCATA GGAAC. Plasmid ds*Venus* was used as a control (T7 Venus 5F′: 5′-TAA TAC GAC TCA CTA TAG GG GACGT AAACG GCCAC AAGTT, T7 Venus 3R′: 5′-CCC TAT AGT GAG TCG TAT TA GGATC TTGAA GTTGG CCTTG). The PCR products were purified using the MEGAclear kit (Ambion). The purified PCR products were used as templates by a MEGASCRIPT T7 transcription kit (Ambion) to produce dsRNA [38]. The dsRNA products were ethanol-precipitated and resuspended in water. The dsRNAs were annealed by incubation at 65 °C for 30 min followed by slow cooling to room temperature. Adjust concentration of the dsRNA were 1–2 μg/μl, and the aliquots were stored at -80 °C.

2.3. Microinjection

Microinjections were performed as described previously [39]. dsRNAs or cRNA were centrifuged, and the top 1–2 μl was used to prepare micro drops from which glass micropipettes were

Please cite this article in press as: H.C. Lee, et al., TRPV3 channels mediate Ca^{2+} influx induced by 2-APB in mouse eggs, Cell Calcium (2015), http://dx.doi.org/10.1016/j.ceca.2015.12.001

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