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TRPV3 channels mediate strontium-induced mouse egg activation

Ingrid Carvacho[1,2], **Hoi Chang Lee**[3], **Rafael A. Fissore**[3], and **David E. Clapham**[1,2,*]

[1]Howard Hughes Medical Institute (HHMI), Department of Cardiology, Boston Children's Hospital, Boston, Massachusetts 02115, USA

[2]Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115, USA

[3]Department of Veterinary and Animal Sciences, University of Massachusetts Amherst, Amherst, Massachusetts 01003, USA

SUMMARY

In mammals, calcium influx is required for oocyte maturation and egg activation. The molecular identities of the calcium-permeant channels that underlie the initiation of embryonic development are not established. Here, we describe a Transient Receptor Potential (TRP) ion channel current activated by TRP agonists that is absent in *TrpV3*$^{-/-}$ eggs. TRPV3 current is differentially expressed during oocyte maturation, reaching a peak of maximum density and activity at metaphase of meiosis II (MII), the stage of fertilization. Selective activation of TRPV3 channels provokes egg activation by mediating massive calcium entry. Widely used to activate eggs, strontium application is known to yield normal offspring in combination with somatic cell nuclear transfer. We show that TRPV3 is required for strontium influx, as *TrpV3*$^{-/-}$ eggs failed to permeate Sr^{2+} or undergo strontium-induced activation. We propose that TRPV3 is the major mediator of calcium influx in mouse eggs and is a putative target for artificial egg activation.

INTRODUCTION

Increases in the intracellular concentration of calcium ($[Ca^{2+}]_i$ initiate a myriad of physiological processes in all cell types, including oocytes and eggs (Berridge et al., 2000; Clapham, 2007). Fully-grown mammalian oocytes are arrested in prophase of meiosis I, also known as the germinal vesicle (GV) stage, until puberty. At this time, an increase in luteinizing hormone (LH) triggers resumption of meiosis (maturation) and progression to the metaphase stage of the second meiosis (MII). This process is known as oocyte maturation. Mature oocytes (eggs) are ovulated and arrested at the MII stage until fertilization. Oocyte maturation is accompanied by an increase in the content of Ca^{2+} stores ($[Ca^{2+}]_{ER}$) and Ca^{2+} influx from the extracellular milieu is required for this increase (Cheon et al., 2013). Oocytes deprived of external Ca^{2+} ($[Ca^{2+}]_e$) or chelation of $[Ca^{2+}]_i$ do not complete meiosis I, suggesting that disruption of Ca^{2+} signaling uncouples the cell cycle machinery (MPF-MAPK) from nuclear maturation (Homa, 1995).

*Correspondence: dclapham@enders.tch.harvard.edu. Phone: 617-919-2680. Fax: 617-731-0787.

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Spermatozoa deliver a male-specific phospholipase C, PLCζ, to the egg that triggers a series of $[Ca^{2+}]_i$ responses that coordinate the exit of MII and progression to the interphase stage, inducing events known collectively as egg activation (Ducibella et al., 2002; Saunders et al., 2002; Schultz and Kopf, 1995). Thus, it is generally accepted that Ca^{2+} influx and intracellular Ca^{2+} release are necessary to complete maturation (Homa, 1995) and to sustain $[Ca^{2+}]_i$ oscillations (Kline and Kline, 1992b) during egg activation. The channels that mediate Ca^{2+} influx during these stages have not been established.

The complement of Ca^{2+} channels expressed in mammalian oocytes has not been completely investigated. Voltage-gated Ca^{2+} channels (Ca_v), consistent with Ca_V3 (T type) Ca^{2+} channels, have been measured in mature mouse eggs (Peres, 1987). During mouse fertilization, changes in the membrane potential are small (Igusa et al., 1983; Jaffe and Cross, 1984) and the oocyte membrane potential is depolarized relative to Ca_V current activation thresholds. Thus, most Ca_V current should be inactivated. In contrast, the relatively voltage-insensitive TRP channels are non-selective, calcium-permeant, channels that function over a much larger range of potentials. In general, TRP channels are modulated by a variety of stimuli and ligands, including G-protein coupled receptors (Ramsey et al., 2006; Venkatachalam and Montell, 2007). TRPV3, a highly temperature-dependent channel with Q_{10}>20 above 32 °C (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002) is most highly expressed on skin and mucosal surfaces, but is also present in dorsal root ganglion, brain, and testis. Here we show that it is also expressed in mouse oocytes and eggs. We found that TRPV3 functional expression increased during oocyte maturation from GV to MII stages. Using mice in which *TrpV3* had been deleted (*TrpV3*$^{-/-}$ or *TrpV3-KO*), we show that specific stimulation of TRPV3 promotes Ca^{2+} entry and induces egg activation.

Mammalian eggs can be parthenogenetically activated by a variety of artificial stimuli, but replacement of external Ca^{2+} by 5–10 mM strontium (Sr^{2+}) has been extensively used to induce activation in rodents (Kline and Kline, 1992a; Liu et al., 2002). This procedure is not associated with chromosome abnormalities (O'Neill et al., 1991) and is capable of promoting full-term development when combined with somatic cell nuclear transplantation (Wakayama et al., 1998). We demonstrate that Sr^{2+} influx during strontium-induced egg activation occurs via TRPV3 channels.

RESULTS

Functional expression of TRPV3 channels in mouse eggs

We used whole-cell patch clamp methods to record from mouse eggs and determine their ion conductances. Prior work established the primary voltage-gated calcium, potassium, and chloride currents in oocytes (Bountra and Martin, 1987; Day et al., 1993; Kolajova et al., 2001; Okamoto et al., 1977; Peres, 1987; Seguin and Baltz, 1997). Here we focus on whether oocytes/eggs have TRP channels.

In response to a voltage ramp (Fig. 1A), addition of the nonselective agent 2-APB (2-aminoethoxydiphenyl borate, 100 μM) evoked an outwardly rectifying current with properties characteristic of TRPV3 (Hu et al., 2004). This current was present in *TrpV3*$^{+/+}$ (*WT*, CD1 strain) and *TrpV3*$^{+/-}$ (heterozygous, *V3-Het*), but absent in *TrpV3*$^{-/-}$ (*V3-KO*) eggs (Fig 1B), confirming the identity of the channel as TRPV3. Fig. 1C summarizes the average current amplitudes. Because *V3-KO* animals used in the initial study were generated from a mixed strain background (Sv129EvTac/C57BALB6) and variations in behavioral responses can be strain-dependent (Huang et al., 2011), we tested responses to the aforementioned agonists in other mouse strains including C57BALB6, Sv129EvTac, CD1, CF1, and the *WT* mixed background Sv129EvTac/C57BALB6. All *WT* strains exhibited similar TRPV3 currents (data not shown). We compared reproductive parameters between

V3-KO, *V3-Het*, and *WT* females, and found no differences in the number of eggs per superovulation (Fig. S1B) or fertility, as reflected by the number of pups/litter (7.4 ± 0.7 for *V3-KO* and *V3-Het*, Fig. S1A).

Since 2-APB is not a selective agent, we next investigated temperature-induced TRPV3 activation in MII eggs (Peier et al., 2002; Smith et al., 2002; Xu et al., 2002). Increasing bath temperature from 23°C to 40°C elicited a large outwardly rectifying whole-cell current (Fig. 1D), which was not present in *V3-KO* eggs (Fig. 1E). As is typical for TRPV3 current (Xu et al., 2002), it rapidly deactivated after removal of the heating stimulus (Fig. 1D). The average heat-activated currents at 40°C, again consistent with TRPV3 properties, were absent in *V3-KO* cells (Fig. 1F). To determine the molecular identity and distribution of TRPV3 on the mouse egg surface, we performed immunofluorescence studies using an anti-TRPV3 antibody. In *WT* eggs, TRPV3 protein was fairly evenly distributed throughout the plasma membrane, but was remarkably absent from the zone overlying the spindle (animal pole, Fig. 1G). *V3-KO* egg surface TRPV3 reactivity was absent, as expected (Fig. 1G). In summary, TRPV3 channels are preferentially located on the vegetal pole of the plasma membrane and are activated by temperature elevation in the physiological range and by the nonselective TRPV3 agonist, 2-APB.

Functional TRPV3 channels progressively increase during oocyte maturation

Since significant changes take place in Ca^{2+} signaling in both oocytes and eggs during maturation (Homa, 1995; Jones et al., 1995; Wakai and Fissore, 2013), we explored changes in TRPV3 functional expression at different oocyte stages. During the GV stage, addition of 100 μM 2-APB did not induce a measurable current (Fig. 2A). Similarly, Ca^{2+} imaging studies at this stage revealed that addition of 200 μM 2-APB failed to trigger an increase in $[Ca^{2+}]_i$ (Fig. 2A, D). Once oocytes had undergone germinal vesicle breakdown (GVBD) and progressed to MI (Fig. 2B), application of 2-APB evoked a TRPV3 current (Fig. 2B) of 23 ± 2 pA/pF (Fig. 2D, *left panel*) and a $[Ca^{2+}]_i$ increase (Fig. 2B, D). At MII (Fig. 2C), when eggs become competent to mount oscillations in response to fertilization, TRPV3 channels responded to 2-APB with a current of 73 ± 6 pA/pF (Fig. 2C, D), which is significantly higher than in MI eggs. Thus, TRPV3 more densely populates the MII membrane compared to the MI membrane (in channels/μm^2). Despite their much smaller surface area (120 ± 7 pF vs. 226 ± 9.5 pF, Fig. 2D) and higher TRPV3 channel density, $[Ca^{2+}]_i$ increases in MII eggs were similar in magnitude to those in MI (Fig. 2C), reflecting the lower sensitivity of Ca^{2+} measurements compared with direct voltage clamp recordings of current (due to native Ca^{2+} buffers and variations in resting membrane potential in unclamped oocytes). Addition of ionomycin, a Ca^{2+} ionophore that under Ca^{2+}-free conditions causes Ca^{2+} release from the intracellular stores, induced a response in all stages of maturation (Fig. 2 A–C, *lower panel*).

Our results show that the number of TRPV3 channels increases during oocyte maturation, being undetectable with electrophysiological recordings in the germinal vesicle (GV) stage and reaching maximal absolute numbers of functional channels in the MII stage (0.1 channels/μm^2 in MII vs. 0.034 channels/μm^2 in MI, assuming an equal open probability, P_o =0.5 for both stages). They also show that ion channel density in oocytes and eggs is very low compared to excitable cells in which these values are typically 10^3–10^4 -fold larger (Hille, 2001).

Carvacrol, a TRPV3 agonist, induces $[Ca^{2+}]_i$ responses and activation in mouse eggs

Carvacrol, the major ingredient in oregano, elicits TRPV3-mediated currents in heterologously expressing cells and in primary keratinocytes (Cheng et al., 2010; Xu et al., 2006). Because *V3-Het* eggs had robust TRPV3 currents in response to 2-APB, we performed functional studies in eggs isolated from heterozygous (*V3-Het)* and knockout

(*V3-KO)* littermates. Addition of 50 μM carvacrol to *V3-Het* eggs evoked a substantial increase in $[Ca^{2+}]_i$ (Fig. 3A, *left panel*) and responded with an additional $[Ca^{2+}]_i$ increase following the addition of 200 μM 2-APB. In contrast, eggs from *V3-KO* mice failed to respond to either agonist (Fig. 3A, *right panel*). We next tested whether stimulation of TRPV3 channel activates mature eggs. Incubation of *WT* and *V3-Het* eggs with 50 μM carvacrol provoked parthenogenesis, as pronuclear (PN) formation was observed within 5 h after carvacrol treatment in roughly half of the mature eggs (Fig. 3B, C). Cleavage to the 2-cell stage was observed 24 h after treatment (67 ± 3% in *WT* eggs and 56 ± 4% in *V3-Het* eggs, Fig. 3B, D). Consistent with Ca^{2+} imaging data, carvacrol failed to activate *V3-KO* eggs (Fig. 3B – D), but did respond to the addition of the Ca^{2+} ionophore, ionomycin (Fig. 4B). Furthermore, incubation of *V3-Het* eggs with 200 μM 2-APB also induced high rates of egg activation, but had no effect on eggs from *V3-KO* mice (Fig. 4A).

TRPV3 channels mediate Sr^{2+} influx and subsequent egg activation

Sr^{2+} has been widely used to induce parthenogenesis in rodent eggs (Kono et al., 1996; Li et al., 2009; Tomashov-Matar et al., 2005). The channel that mediates Sr^{2+} influx has not been reported in any species, and whether Sr^{2+} and Ca^{2+} permeate the same channel(s) in mouse eggs is unknown. To ascertain whether the TRPV3 channel mediates Sr^{2+} influx, we recorded the heat-activated TRPV3 current in *V3-Het* and *V3-KO* cells in the presence of an extracellular solution containing 10 mM $SrCl_2$ and nominal $[Ca^{2+}]$ (~10 μM) (Fig. 5A). At −80 mV, the current measured in *V3-Het* cells averaged −1.4 ± 0.1 pA/pF, while the current in *V3-KO* cells was 10-fold lower (−0.14 ± 0.1 pA/pF). Similarly, at +80 mV, *V3-Het* current averaged 36 ± 2 pA/pF, 36-fold larger than *V3-KO* current (1.0 ± 0.06 pA/pF; data not shown). Reversal potentials were consistent with Sr^{2+} and Na^+ permeation in *V3-Het* eggs (E_{rev} ~ −15 mV) and a lack of permeability in *V3-KO* eggs (E_{rev} <−60 mV; Fig. 5A). The absence of inward Sr^{2+} current in *V3-KO* eggs correlates with the lack of $SrCl_2$-induced oscillations in these eggs (Fig. 5B) and their inability to become activated in media supplemented with $SrCl_2$, as assessed by 2-cell formation (Fig. 5C,D). Nearly all *WT* eggs (89 ± 11 %) and over half of *V3-Het* eggs (61 ± 5.3 %) became activated in these experiments.

TRPV3-mediated Ca^{2+} influx is not required for maintenance of calcium oscillations

The fertilization-associated $[Ca^{2+}]_i$ oscillations that underlie activation of mammalian eggs are thought to be triggered by the sperm-specific PLCζ. PLCζ activates the egg's phosphoinositide pathway to generate IP_3, which in turn gates the endoplasmic reticular calcium-permeant IP_3R (Miyazaki and Ito, 2006; Saunders et al., 2002). While the first few oscillations of Ca^{2+} rely on internal Ca^{2+} stores, the persistence of the Ca^{2+}oscillations requires Ca^{2+} influx (Igusa and Miyazaki, 1983; Kline and Kline, 1992b; Winston et al., 1995). To ascertain whether TRPV3 channels are required for these oscillations in response to fertilization, we tested sperm-induced calcium oscillations in *V3-KO* and *V3-Het* MII eggs. We observed the first $[Ca^{2+}]_i$ transients 5–10 min after addition of sperm (Fig. 6A; see (Swann, 2013)). No difference was observed between the two groups during 2.5 h of recording. Confirming the *in vivo* data (Fig. S1A), no differences were found for *in vitro* fertilization success, assessed as the rate of 2-cell formation between *V3-KO* and *V3-Het* eggs (Fig. 6B). We next examined whether the activity of TRPV3 channels was required to support oscillations induced by injection of PLCζ cRNA. These oscillations, which were monitored for 6 h, were similar in *V3-KO* and *V3-Het* MII eggs with respect to time to initiation, amplitude, frequency and duration (Fig. 6C). These data suggest that PLCζ1 does not substantially potentiate TRPV3 as a mechanism of its action on Ca^{2+} oscillations. However, addition of 200 μM 2-APB to *V3-Het* eggs that were undergoing oscillations at 37°C dramatically increased Ca^{2+} influx and prevented $[Ca^{2+}]_i$ from returning to baseline, immediately blocking oscillations in *V3-KO* eggs (Fig. 6C). Finally and as expected, the

addition of the TRPV3 agonist, carvacrol (50 μM), to oscillating *WT* eggs increased Ca^{2+} influx and immediately halted the oscillations. Addition of carvacrol to oscillating *V3-KO* eggs had no effect in the pattern or frequency of the Ca^{2+} oscillations (Fig. S2A).

Examination of Compensatory Mechanisms for loss of TrpV3—The lack of an effect of TRPV3 deletion on fertilization-induced oscillations could be due to compensatory Ca^{2+} influx by other channels. One possibility is that other TRP channels were upregulated in *V3-KO* mice. If this were the case, we should have observed them in our recordings. Small Ca_V3 (T type) currents (~5 pA/pF) are present in all the oocyte stages studied here, and previously identified as Cav3.2 (Kang et al., 2007). *Cav3.2 KO* animals do not display a fertility phenotype (Chen et al., 2003) and following *in vitro* fertilization, *Cav3.2-KO* eggs [Ca^{2+}] oscillated at normal frequencies and durations (M. Bernhardt et al., 2013, Soc. for the study of Reproduction (SSR), abstract #405). In agreement with these data, the calcium channel blocker, mibefradil (20 μM) did not prevent *in vitro* oocyte maturation in WT and *V3-KO* oocytes, and PLCζ injection still activated these eggs (data not shown).

When $[Ca^{2+}]_{ER}$ is severely depleted, the Stim/Orai complex is assembled to mediate CRAC current in many cells (Lewis, 2011). The contribution of CRAC/SOCE channels to Ca^{2+} homeostasis in mouse oocytes and eggs is controversial. Thapsigargin, which blocks SERCA pumps and thus activates CRAC, prematurely terminated oscillations, and was assumed to reflect CRAC activation in this process (Kline and Kline, 1992b). However, Ca^{2+} overload alone, as we observed with TRPV3 stimulation, also terminates Ca^{2+} oscillations. Recent studies suggest that CRAC channels are not functional in mouse eggs (Miao et al., 2012; Takahashi et al., 2013). We evaluated the pattern of PLCζ-induced calcium oscillations in *V3-KO* and *Het-V3* eggs in the presence of the CRAC blocker STA-12-5775 (RO2959; 3μM (Chen et al., 2013)). In both groups of eggs, the frequency of calcium oscillations after the addition of the blocker was unaltered, showing no compensation of TRPV3 channel function by CRAC channels (data not shown).

DISCUSSION

Here we examined the contribution of a TRP channel to Ca^{2+} influx in mouse oocytes and eggs during maturation and fertilization. Using voltage clamp and calcium imaging measurements of *WT* and *V3-KO* mouse oocytes, we show that TRPV3 becomes the major calcium entry pathway in these cells as they mature. Calcium permeation via TRPV3, as induced by temperature elevation, and the TRPV3 agonists 2-APB and carvacrol, were confirmed using TRPV3 knockout mice. Notably, TRPV3 channels are the major mediators of $SrCl_2$ influx during strontium-induced activation in mouse eggs. $SrCl_2$ failed to induce oscillations or egg activation in TRPV3-null eggs.

The small T-type, CaV3.2, currents in eggs do not appear to be necessary or sufficient for calcium oscillations. In any case, the normally depolarized egg membrane potential insures that they are primarily inactivated (see also (Okamoto et al., 1977; Peres, 1987). Thus, Ca_v channels would be most likely be important during an acute depolarizing stimulus, such as fertilization in some species (Whitaker, 2006). Interestingly, commonly used Ca_v1 (L-type) and Ca_v3 (T-type) channel-blocking drugs are not known to be associated with human female infertility.

Another potential source of calcium entry, CRAC currents, might be active at the GV stage of mouse oocytes, but their function decreases as maturation progresses (Cheon et al., 2013; Lee et al., 2013). Expression of Stim1 and Orai1 was detected in mouse oocytes and eggs (Cheon et al., 2013) but to date, no Stim1 or Orai1 subtype female heterozygous or KO mice are reported to be subfertile. Since $Orai1^{-/-}$ and $Stim1^{-/-}$ mice died perinatally, tissue- and

age-specific genetic deletion would clarify this issue. Direct measurements of CRAC current under voltage clamp would likely clear up these inconsistencies, but the size of eggs makes this task difficult due to the size of background currents. In our studies, if CRAC currents are present, they are ≤ 3 pA/pF (in 20 mM $[Ca^{2+}]_e$,), but still large enough to be significant in maintaining cytoplasmic and ER levels. We found that addition of a specific CRAC (Orai1, Orai3) channel blocker during *in vitro* maturation of *Het-V3* and *V3-KO* oocytes did not affect GVBD, although we observed a slight decrease in the percentage of polar body extrusion in both groups. Further experiments, such as tissue-specific deletion of Orai subunits, are necessary to clarify the function of CRAC channels during maturation.

Functional expression of TRPV3 channels during mouse oocyte maturation

At the germinal vesicle stage of oocyte development, TRPV3 currents were undetectable and, consistent with this observation; conditions that activate TRPV3 fail to induce Ca^{2+} influx. By the MI stage, however, small TRPV3 currents were recorded and these became significantly larger (in both net amplitude and density) in MII oocytes. Thus during maturation, increases in TRPV3 functional expression coincides with meiotic progression during maturation. $[Ca^{2+}]_e$ is required for progression beyond GVBD during oocyte maturation (Wakai and Fissore, 2013); without $[Ca^{2+}]_e$ the formation of the polar body is inhibited and oocytes fail to progress past MI (Tombes et al., 1992). *In vivo*, the immature GV stage oocyte maintains direct communication with the surrounding cumulus and granulosa cells through gap junctions, permitting heterologous metabolic and electrical coupling (Homa, 1995). However, spontaneous calcium oscillations recorded in GV stage oocytes are not dependent on the surrounding follicle cells (Carroll and Swann, 1992).

Although TRPV3 currents coincide with meiotic progression during maturation, the TRPV3 channel function is not required for this process (data not shown). The striking fact is that *V3-KO* females mice have normal fertility, number of offspring per litter, and the mice have only minor phenotypes related to skin and hair rather than mating or reproduction (Cheng et al., 2010). Constitutively active TRPV3 mutations cause hair loss and increase susceptibility to dermatitis and inflammatory skin lesions in rodents (Asakawa et al., 2006; Yoshioka et al., 2009), although without apparent defects in female fertility. The Olmsted syndrome, a rare human congenital disorder producing palmoplantar keratoderma, alopecia, and severe itching, is associated with TRPV3 gain-of-function mutations (Lin et al., 2012). However, it is unclear whether female fertility is affected.

Stimulation of TRV3 channels leads to egg activation

Activation of TRPV3 by carvacrol and 2-APB induces calcium entry, which can parthenogenetically activate eggs. Treatment with calcium ionophores such as ionomycin or A213187 can result in embryos with decreased or impaired developmental competence. The long-term ramifications on offspring are unknown (Swain and Pool, 2008), even though these manipulations are used in patients who have low fertilization potential (Heindryckx et al., 2005; Nasr-Esfahani et al., 2010). Thus, the understanding of TRPV3 expression and function in human eggs may be an opportunity to improve artificial oocyte activation (AOA) via specific activation of TRPV3 channels rather than the use of nonspecific ionophores.

Sr^2-induced egg activation and TRPV3 channels

Strontium induces egg activation by promoting oscillations in $[Ca^{2+}]_i/[Sr^{2+}]$, perhaps by sensitizing the egg IP_3R1 receptors. These $SrCl_2$-induced calcium oscillations are distinct from those provoked by the sperm (or by adenophostin, a nonhydrolyzable agonist of the IP_3R) and do not induce down regulation of the IP_3R (Brind et al., 2000; Jellerette et al., 2000). The mechanism of Sr^{2+} gating of the IP_3R1 is thus the TRPV3 channel, since it mediates Sr^{2+} influx into rodent eggs. It has been suggested that Sr^{2+} could sensitize IP_3Rs

and facilitate calcium oscillations (Zhang et al., 2005), perhaps by substituting for calcium in the potentiation of IP_3Rs (Bezprozvanny et al., 1991; Girard and Clapham, 1993; Marshall and Taylor, 1994; Stehno-Bittel et al., 1995). Strontium-induced egg activation is mediated by CaMKIIγ as CaMKII$\gamma^{-/-}$ eggs failed to respond to Sr^{2+} treatment, likely due to a failure to resume meiosis II (Backs et al., 2010).

The fact that *V3-KO* eggs do not to respond to Sr^{2+} is clear evidence that under the conditions in which Sr^{2+}-induced activation was measured (37°C, 10 mM $SrCl_2$ in the extracellular media, ~10 μM [Ca]), TRPV3 is required for measurable strontium permeation. This does not mean that TRPV3 is the only strontium-permeable channel in the egg plasma membrane. In fact, Ca_V channels, present in the egg plasma membrane, are permeable to Sr^{2+} as shown by Hirano *et al*. (Hirano et al., 1989a; Hirano et al., 1989b), but Ca_V3 channels are largely inactivated at egg resting membrane potentials. Since the egg membrane potential remains stable at potentials in which Ca_Vs are inactivated (Igusa et al., 1983), we did not observe measurable Sr^{2+} permeation via Ca_V, positioning TRPV3 as the major mechanism for Sr^{2+}-induced egg activation in mice.

Regulation and function of TRPV3 channels in mouse oocytes and eggs

Muscarinic receptors couple to the $G_{q/11}$ proteins in mouse eggs to activate PLCβ (Igarashi et al., 2007; Williams et al., 1998) and initiate $PI(4,5)P_2$ hydrolysis. This, in turn, generates IP_3 and the PKC activator, diacylglycerol (Clapham, 2007). Muscarinic agonists induce membrane potential changes (Eusebi et al., 1979) and a series of small $[Ca^{2+}]_i$ oscillations in mouse eggs (Swann, 1992). Stimulation of PKC activity greatly enhances the frequency of these oscillations (Halet et al., 2004). These same pathways also modify TRPV3 channels: in somatic cells, carbachol (Xu et al., 2006), diacylglycerol, phorbol-12-myristate-13-acetate (Hu et al., 2006), and PLC-coupled receptor-catalyzed $PI(4,5)P_2$ hydrolysis (Doerner et al., 2011) all modulate TRPV3 activity. Ca^{2+}, via calmodulin, also activates CaMKII to modulate oscillatory changes during fertilization (Markoulaki et al., 2003) and govern exit from MII (Ducibella and Fissore, 2008). Ca^{2+}/calmodulin initially inhibits TRPV3 activity, perhaps explaining sensitization of TRPV3 to repetitive stimulation by mediating Ca^{2+}-dependent initial channel inhibition (Xiao et al., 2008). In summary, there is good evidence that PLC activation regulates both oocyte and egg TRPV3 and $[Ca^{2+}]_i$.

In order to test modulation of TRPV3 activity by hormones, we patch-clamped MII eggs in the presence of progesterone (30 μM), estradiol (100 μM), and prostaglandin E2 (1 μM). None of these had an effect on TRPV3 current at RT (data not shown).

PLCζ, cloned from a mouse spermatid cDNA library, is unlike PLCβ in that it is soluble, has no pleckstrin homology domain, and is active at resting (100 nM) $[Ca^{2+}]$ (Kouchi et al., 2004). PLCζ is expressed only in males. Injection of PLCζ cRNA results in egg Ca^{2+} oscillations that are prevented by inhibition of protein synthesis (Saunders et al., 2002). Since male-specific PLCζ more effectively generates $PI(4,5)P_2$ production in eggs (Swann and Lai, 2013), we would expect that PLCζ overexpression would potentiate endogenous egg TRPV3 current. In spite of the expression of TRPV3 in MII mouse eggs and its predicted nearly constitutive function at 37°C, TRPV3-mediated Ca^{2+} influx is seemingly dispensable for maintaining PLCζ-induced $[Ca^{2+}]_i$ oscillations or for fertility, as *V3-KO* females show normal oscillatory responses in eggs and after fertilization.

What is the function of TRPV3 in oocytes and eggs? One possibility is that TRPV3 channels constitutively conduct low levels of Ca^{2+} to maintain Ca^{2+} homeostasis. Nevertheless, given the importance of these functions, eggs may have a redundant system that may explain the unchanged oscillation pattern and fertility in *V3-KO* eggs. It is likely that an unknown G protein receptor may be activated to induce PLCβ activation and modulate TRPV3 activity.

Less probably, but more intriguing, is that highly temperature-sensitive TRPV3 may function to 'reawaken' egg maturation/activation in mammals that significantly lower their body temperatures during hibernation, and that TRPV3 is thus not functionally relevant in mammals that do not hibernate. Increasing ambient temperatures above 32°C would increase TRPV3 activity and prime progression to MII. Finally, it is possible that there are unknown endogenous direct activators of oocyte and egg TRPV3. Future studies will examine the expression of TRPV3 in other species, such as humans, as well as the regulation of TRPV3 function.

EXPERIMENTAL PROCEDURES

Oocyte collection

Six-to-ten-week-old females (CD1, *TrpV3*$^{-/-}$ colony (Cheng et al., 2010)) were superovulated with intraperitoneal (i.p.) injection of 5 IU pregnant mare's serum gonadotropin (PSMG, Calbiochem, EMD Biosciences), followed 48 h later by i.p. injection of 5 IU of human chorionic gonadotropin (hCG, Calbiochem, EMD Biosciences). Ovulated eggs (cumulus masses) were obtained by pulling the oviducts open with fine forceps in a HEPES-buffered culture medium (M2 medium, Millipore) 13–16 h after administration of hCG. Cumulus cells were removed using hyaluronidase (Calbiochem, EMD Biosciences) and gentle aspiration through a pipette. The *zona pellucidae* (ZP) were removed by exposure to Tyrode's acid solution (pH 2.5) for a few seconds followed by thorough washing in M2 medium. GV oocytes were collected from the ovaries of 5- to 12-week-old CD-1 female mice. Females were injected with 5 IU PMSG and cumulus cell-enclosed oocytes were recovered 42–46 h later into HEPES-buffered culture medium (M2 medium) and 100 μM isobutyl-1-methylxanthine (IBMX). Cumulus cells were removed by repeated pipetting and denuded oocytes were matured in HTF medium at 37°C in 5% CO_2. After 5–6 hours of in vitro maturation, eggs were in MI. All animal experiments were approved by the Boston Children's Hospital Institutional Animal Use and Care Committee (Protocols #13-03-2380R and 11-01-1879).

Electrophysiology

Whole-cell currents were measured at 22–24°C using an Axopatch 200B amplifier digitized at 20 kHz (Digidata 1320A) and filtered at 5 kHz Electrophysiology recordings were performed on the same day of egg isolation 8 hours post-surgery. Eggs were maintained in human tubal fluid medium (HTF, EMD Millipore) at 37°C and 5% CO2. For temperature ramps, the perfusate was heated using a TC-324B temperature controller and SH-27B solution heater (Warner Instrument Corporation). Data were analyzed using Clampfit (Molecular Devices) and Origin 7.0 (OriginLab). Pipettes of 1–3 MΩ resistance were made from glass capillaries (593600, A–M systems). Series resistance was compensated by 60–80%. The intracellular solution contained (in mM): 152 Cs-Methanesulfonate, 1 mM Cs-BAPTA, 10 HEPES, 2 MgATP, 8 NaCl, 0.3 NaGTP, pH: 7.3–7.4. The external solution contained (in mM): 125 NaCl, 6 KCl, 20 mM $CaCl_2$, 1.2 $MgCl_2$, 20 mM HEPES-NaOH, pH: 7.3–7.4. The response to 2-aminoethoxydiphenyl borate (2-APB, Sigma) were measured in an external solution containing (in mM): 140 mM NaCl, 10 mM HEPES, 10 mM glucose, 4 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$. In order to avoid chloride currents (Qu and Hartzell, 2000) a solution of Na-gluconate was used in the temperature response experiments and in the Sr^{2+} permeability experiments. The osmolarity of all solutions was 290–300 mOsm. All voltages were corrected for measured junction potentials present between the internal and external solution before seal formation. TRPV3 currents were activated by voltage ramps from −100 mV to 100 mV (600 ms, every 2 s), in the presence of 2-APB or temperature ramps. The holding potential (HP) was −80 mV. For Sr^{2+}

permeability recordings and to avoid T-type voltage gated Ca^{2+} channels, ramps were from +100 mV to −100 mV with a HP of 0 mV.

$[Ca^{2+}]_i$ imaging

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was estimated using the Ca^{2+} sensitive dye Fura-2AM (Life Technologies). Briefly, oocytes/eggs were loaded with 1.25 μM Fura-2AM supplemented with 0.02% pluronic acid (Life Technologies) for 30 min at room temperature. To estimate $[Ca^{2+}]_i$, oocytes/eggs were thoroughly washed and attached to glass-bottom chambers. Responses to TRPV3 agonists were recorded in FCS-free (serum free) HEPES-buffered Chatot, Ziomek, and Bavister (HCZB) medium. For Figures 2, 5B, 6C, and S2A., the Ca^{2+} measurements were performed on a Nikon Diaphot microscope fitted for fluorescence measurements. The objective used was a 20x Nikon Fluor and the excitation lamp was a 75 W Xenon lamp. Emitted light >510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scienti c) using SimplePCI (C-Imaging Systems). In Fig. 3A and 6A, cells were alternatively illuminated with 340 and 380 nm light (Lambda DG-4; Sutter Instrument Co.) for 75–100ms, emission light >510 nm was captured by CCD camera. The setup was equipped with a 175 W Xenon lamp; objective 20x UApo/340. Data was analyzed with Slidebook software (Intelligent Imaging Innovations) after background subtraction. All fura-2AM ratios were normalized.

Parthenogenetic egg activation

For TRPV3-mediated egg activation, eggs were incubated at 37°C for 10 min in 50 μM carvacrol in CZB medium or for 30 min in 200 μM 2-APB in modified Krebs–Ringer bicarbonate medium. In both cases, the media was FCS-free 0.1% PVA (Poly-vinyl alcohol, Sigma). In studies using *V3-KO* eggs, activation was induced by exposure to 2.5 μM ionomycin (A.G. Scientific) for 5 min in Ca^{2+}-free CZB media followed by incubation for 4h in CZB (0.1% PVA) supplemented with cycloheximide (CHX, 20 μg/ml, EMD Biosciences). All procedures were in humidified 5% CO_2 at 37°C. For Sr^{2+} activation, eggs were incubated eggs for 2 h in Ca^{2+}-free-CZB medium supplemented with 10 mM $SrCl_2$. Eggs were then washed and transferred to HTF medium and cultured to the 2-cell stage. Eggs were evaluated for signs of activation 5 and 24 h after treatment under phase contrast microscopy. Activated eggs were classified according to the following criteria: (1) PN group, consisted of zygotes forming a single PN with first and second polar bodies (5 h post-treatment); (2) cleaved group; eggs undergoing immediate cleavage after 24 h. Eggs without 2^{nd} polar bodies, PN formation, or those failing to cleave were considered as unactivated (MII egg). Fragmented eggs were excluded from analysis.

Microinjections

Oocytes were microinjected as described (Kurokawa et al., 2005). Reagents were loaded onto glass micropipettes and 7–12 pL (1–3% of the total volume of the egg) delivered by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus). The full-length of mouse PLCζ, cDNA, a kind gift from Dr. K. Fukami (Tokyo University of Pharmacy and Life Science, Japan) was subcloned into pcDNA6/ myc-His (Invitrogen) for in vitro transcription. Plasmids were linearized and the cDNA was *in vitro* transcribed using the T7 mMESSAGEmMACHINEKit (Ambion).

See Supplemental Methods for *Sperm isolation, IVF, and Immunofluorescence staining*

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- TRPV3 channels are functionally expressed in mouse oocytes and eggs.
- TRPV3 channels are progressively expressed during oocyte maturation.
- TRPV3 channels can mediate Ca^{2+} influx and parthenogenesis in MII eggs.
- Strontium influx and subsequent egg activation in mice requires TRPV3 channels.

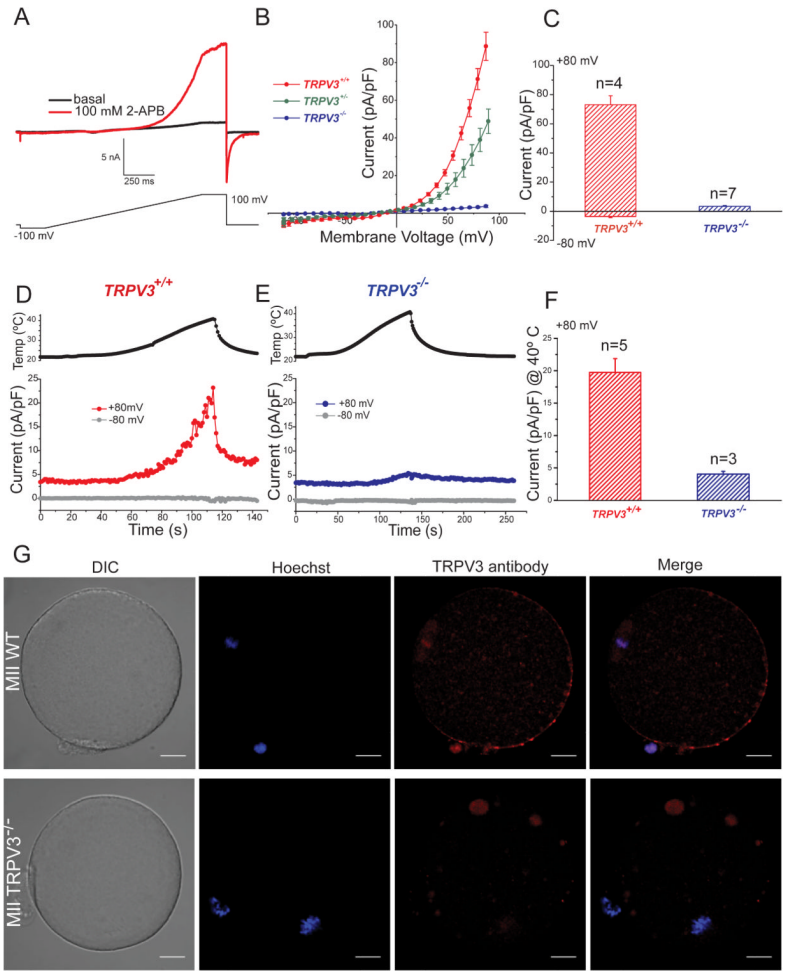

Figure 1.

TRPV3 channels are expressed in MII mouse egg. **A–F**. Whole-cell voltage clamp recordings from an MII mouse egg. **A**. Current evoked from a voltage ramp from −100 to +100 mV in the absence (*black trace*) and presence (*red trace*) of 100 μM 2-APB. **B**. Current-voltage relations (I–Vs) in response to 100 μM 2-APB in *WT* (*TrpV3*$^{+/+}$, CD-1 strain), heterozygous (*TrpV3*$^{+/−}$) and eggs lacking TRPV3 (*TrpV3-KO*, *TrpV3*$^{−/−}$). **C.** Averaged 2-APB evoked current recorded at +80 mV and −80 mV in *WT* and *V3-KO* eggs (73 ± 6 pA/pF for *WT* and 3.3 ± 0.5 pA/pF for *V3-KO* at +80 mV; −3.5 ± 0.8 pA/pF for *WT* and −0.2 ± 0.04 pA/pF for *V3-KO* eggs at −80 mV (± S.E.M). **D–E**. Temperature responses for *WT* and *V3-KO* eggs. *Upper panels*: Temperature (egg surface). *Lower panels*: Current responses at +80 mV (red trace) and −80 mV (gray trace) for *WT* and *V3-KO* eggs. **F**. Average TRPV3 current in response to 40°C recorded at +80 mV for *WT* and *KO* eggs. Current was 20 ± 2 pA/pF in *WT* eggs in contrast to only 4 ± 0.4 pA/pF in *V3-KO* eggs. The averaged inward current at −80 mV was −0.6 ± 0.2 pA/pF for *WT* and −0.4 ± 0.08 pA/pF for *V3-KO* eggs (data not shown). ± S.E.M; # of experiments are indicated over the bars. **G**. TRPV3 protein localization in mature mouse MII zona-free eggs; shown are differential interference contrast (DIC), DNA staining (Hoechst), and anti-TRPV3 staining. Upper panel: *WT* (129SvEvTac strain), *Lower panel: TrpV3-KO* egg. Most of the antibody-stained protein is at the membrane, except at the animal pole. Scale bar: 10 μm.

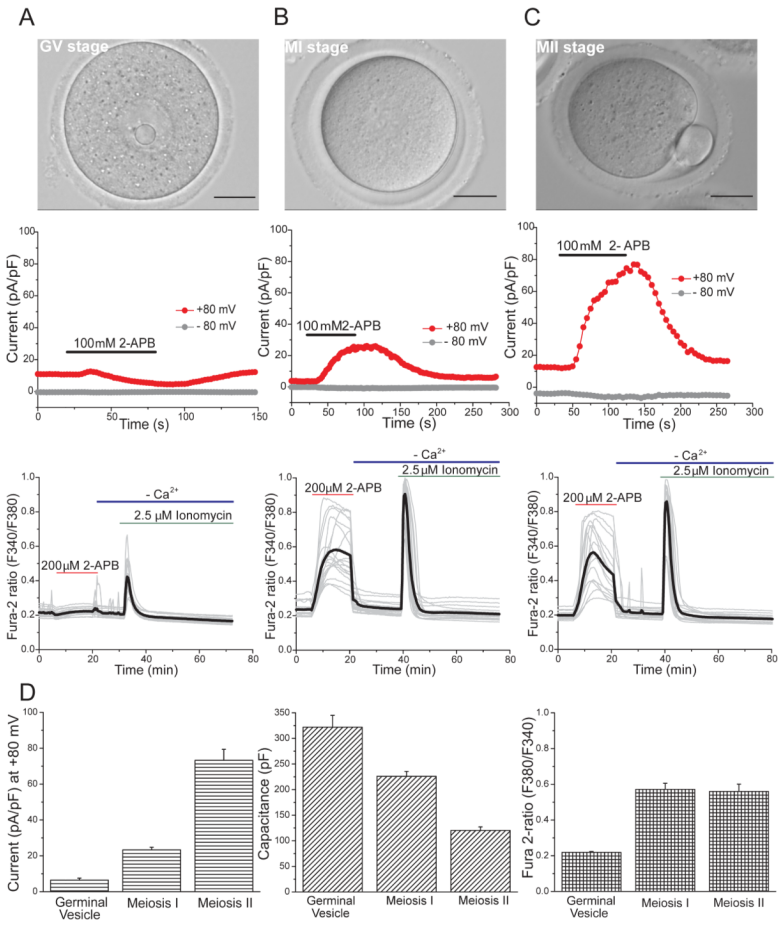

Figure 2.

I_{TRPV3} in response to 2-APB during oocyte maturation. **A–C**. *Upper panels*: Germinal vesicle (GV) oocyte, Meiosis I (MI) and Meiosis II (MII) eggs. Scale bar: 50 μm. *Middle panel*: Whole-cell patch -clamp recording in response to 100 μM 2-APB (*black bar*). *Lower panel*: Changes in $[Ca^{2+}]_i$ induced by 200 μM 2-APB (*red bars; black= averaged trace.* GV, n=19; MI, n=19; MII, n=16) and ionomycin (*green bars*) in nominal Ca^{2+}-free solutions (*blue bars*). **D**. Summary of parameters measured at different stages of oocyte maturation. *Left panel*: peak current measured at +80 mV in response to 100 μM 2-APB (GV, n=4; MI, n=3; MII, n=4). *Middle panel*: Whole-cell capacitance measurements (pF) (GV, n=4; MI, n=5; MII, n=10). *Right panel*: Peak fura-2 ratio, reflecting relative $[Ca^{2+}]_i$. Data are averages ± S.E.M. Scale bar: 50 μm.

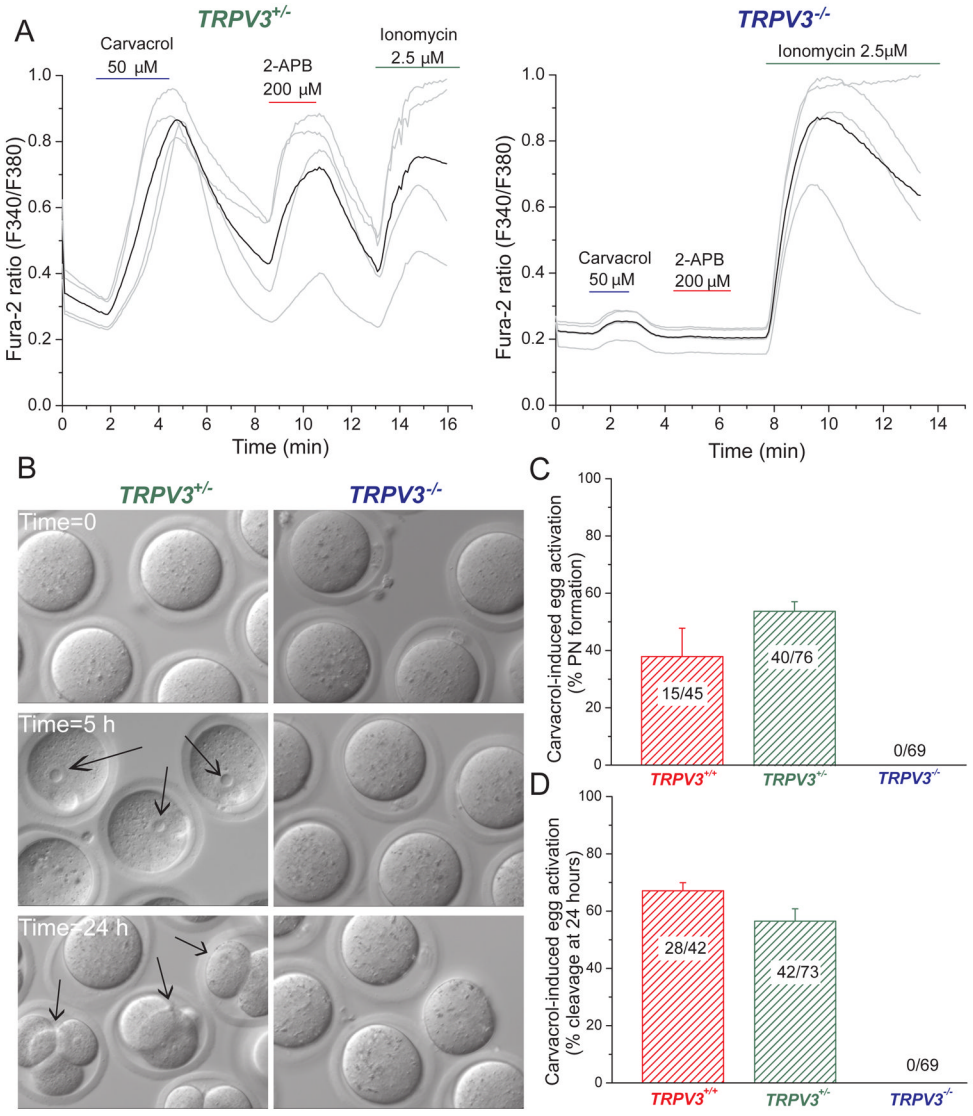

Figure 3.

The TRPV3 channel agonist, carvacrol, induces Ca^{2+} responses and activation in MII mouse eggs. **A**. Changes in $[Ca^{2+}]_i$ induced by TRPV3 channels activated by 50 μM carvacrol (*violet*), 200 μM 2-APB (*red*), and ionomycin (*green*) in *TrpV3*$^{+/-}$ and *TrpV3*$^{-/-}$ cells (*V3-Het*, n=4; *V3-KO*, n=4). **B.** Activation of *TrpV3*$^{+/-}$, but not *TrpV3*$^{-/-}$ eggs, by treatment with 50 μM carvacrol (37°C, 10 min). Arrows indicate PN formation (5 h, left panel) and cell cleavage (24 h, left panel). **C.** Percentages of PN formation in *WT* (*TrpV3*$^{+/+}$, CD1 strain), heterozygous (*TrpV3*$^{+/-}$) and *V3-KO* (*TrpV3*$^{-/-}$) eggs 5–6 h after of carvacrol activation. Numbers of eggs undergoing PN formation/total number of eggs is indicated. **D**. Percentage of eggs cleaved after 24 h exposure to carvacrol. Numbers of 2, 3, or 4-cell blastomeres over the total number of eggs is indicated. Data are averages ± S.E.M.

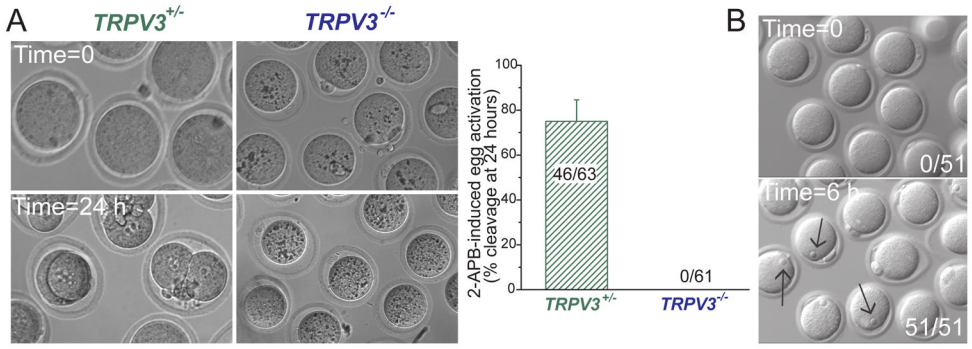

Figure 4.

2-APB induces egg activation in *TrpV3*$^{+/-}$ eggs but not in *TrpV3*$^{-/-}$ MII eggs. **A.** Treatment with 2-APB (200 μM; 37°C) for 30 min activates eggs. *Left panel*: 2-APB induces cleavage, observed 24 h after 200 μM 2-APB treatment in *TrpV3-Het* MII eggs, but not in *TrpV3*-KO eggs. *Right panel*: Percentage of eggs undergoing cleavage at 24 h. Numbers of 2, 3, or 4-cell blastomeres over the total number of eggs is indicated. Data are averages ± S.E.M. **B.** Ionomycin (2.5 μM for 5 min) at 37°C induces activation of *TrpV3-KO* eggs. Numbers of eggs with PN formation/total number of eggs is indicated. Arrows indicate PN formation (right panel).

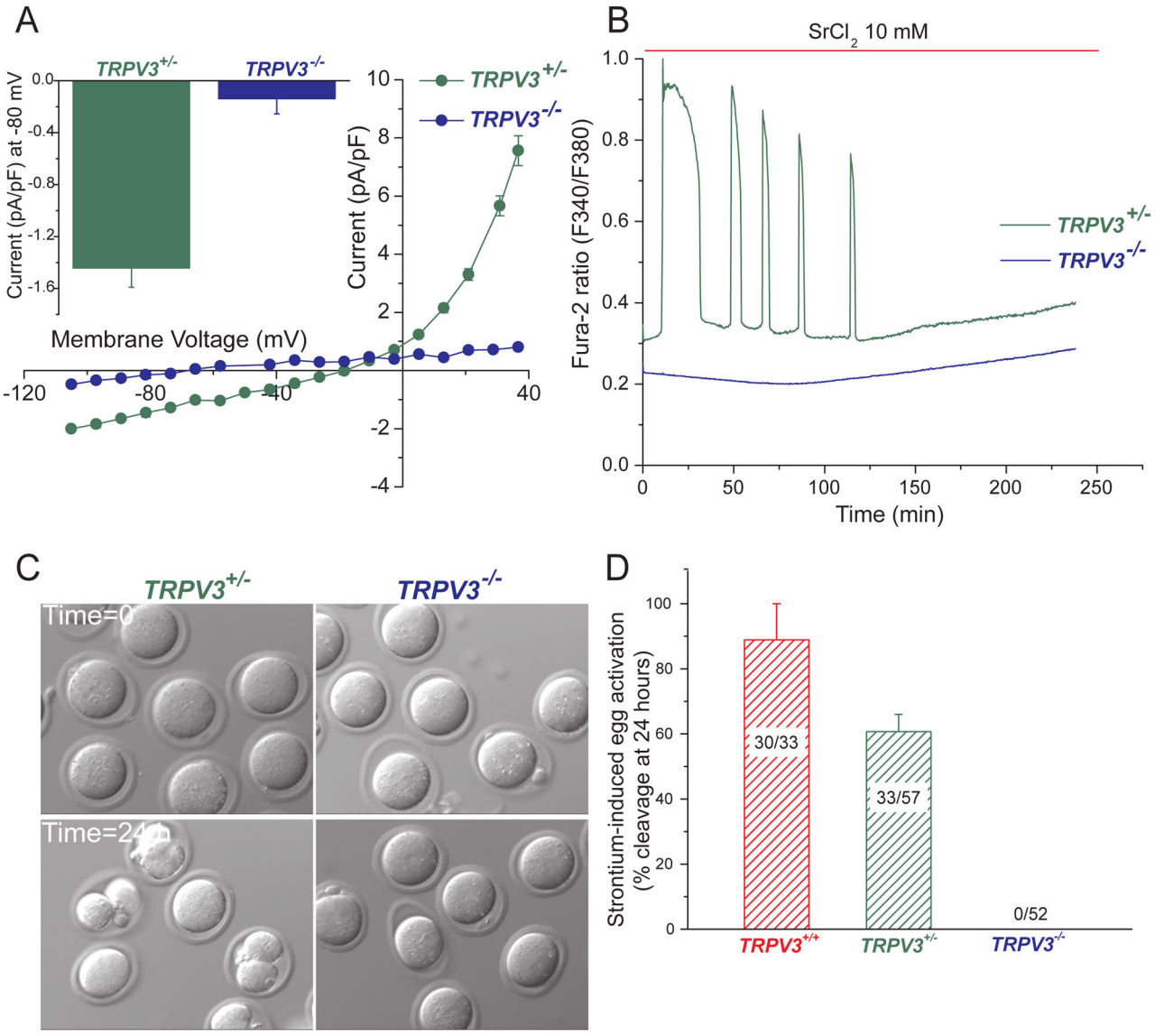

Figure 5.

TRPV3 channels mediate Sr^{2+} influx and subsequent egg activation. **A**. Results of whole-cell patch -clamp recording (37°C) of MII eggs from heterozygous (*TrpV3*$^{+/-}$, *green symbols*, n=4) and *TrpV3-KO* (*TrpV3*$^{-/-}$, *blue symbols*, n=4) mice. Current-voltage (I–V) relation in 10 mM $SrCl_2$. *Inset*: Averaged current at −80 mV. **B**. Oscillations induced by Sr^{2+} in a *TrpV3*$^{+/-}$ egg (*green line*, n=6), but not in a *TrpV3-KO* egg (*blue line*, n=8). **C**. Sr^{2+} induces cleavage in *V3-Het* (*left*) but not in *V3-KO* (*right*) eggs. **D**. Percentage of cleaved eggs after 24 h of 10 mM Sr^{2+} treatment. Numbers of 2, 3, or 4-cell blastomeres/ total number of eggs is indicated. Data are averages ± S.E.M.

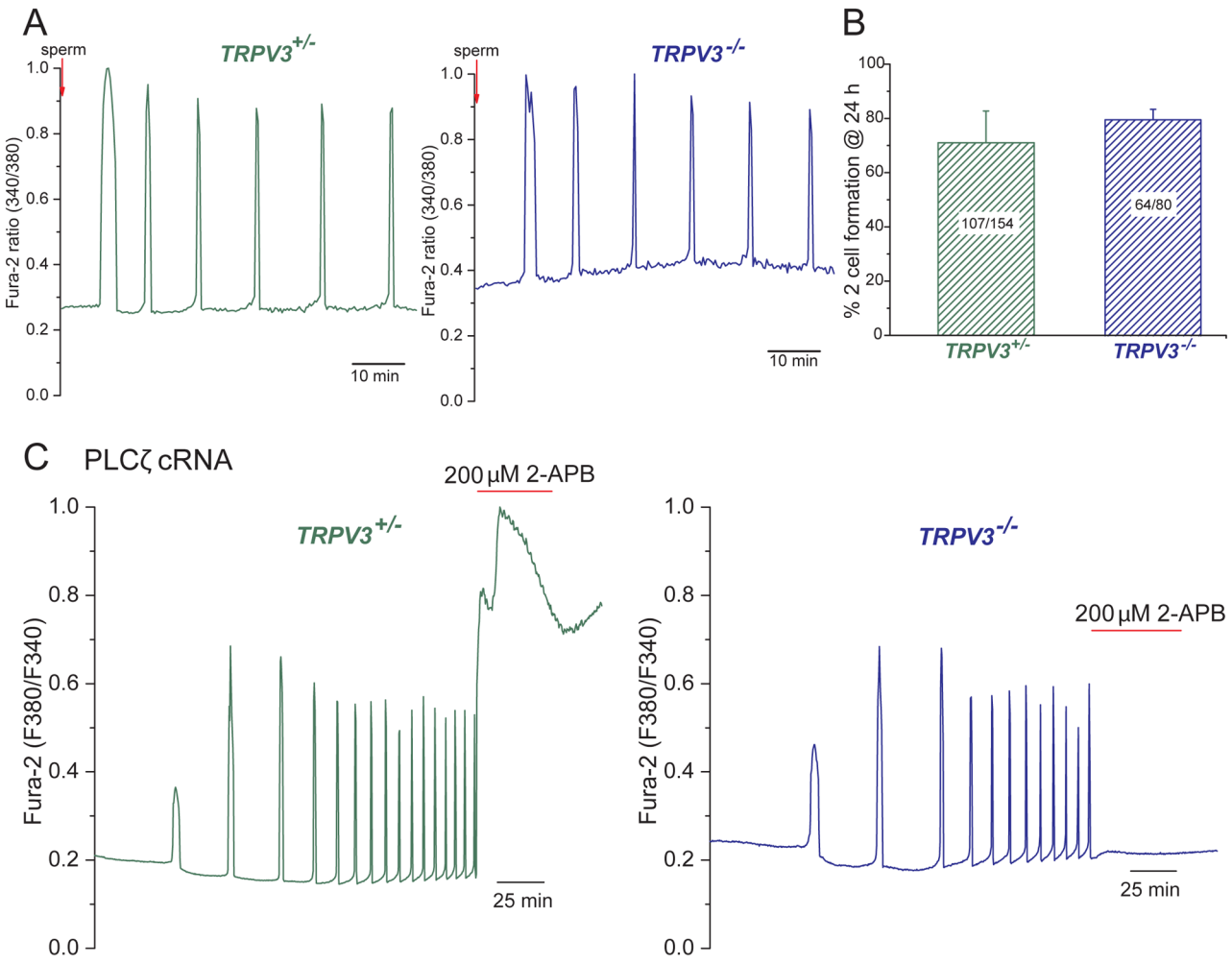

Figure 6.

Calcium influx through TRPV3 channels does not contribute to early development in mouse eggs. **A.** Oscillations induced by sperm fertilization in heterozygous (left, *TrpV3*$^{+/-}$, *green line*, n=12/24 have 5–6 oscillations in 60 min) and *V3-KO* (*right*) eggs (*blue line*, n=9/18 have 5–6 oscillations in 60 min). **B.** *In vitro* fertilization (IVF) rates measured by 2-cell formation after 24 h of fertilization showed no difference between *V3-Het* and *V3-KO* eggs (71 ± 12 for *V3-Het* vs. 80 ± 4 for *V3-KO* eggs, $p > 0.05$). Numbers of 2-cell blastomeres/total number of eggs is indicated. Data are averages ± S.E.M. **C.** $[Ca^{2+}]_i$ responses were induced by injection of 0.01µg/µl mPLCζ cRNA (similar responses were obtained by 0.05 µg/µl mPLCζ cRNA injection). $[Ca^{2+}]_i$ oscillations (37°C) in a control egg (*TrpV3*$^{+/-}$, *left panel*, n=8) and *KO* egg (*TrpV3*$^{-/-}$, *right panel*, n=3). 200 µM 2-APB (*red bar*) was applied at the end of the experiment.