The Xenopus TRPV6 Homolog Encodes a Mg$^{2+}$-Permeant Channel That is Inhibited by Interaction With TRPC1

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The TRP gene family encodes primarily cation non-selective, Ca$^{2+}$ permeant channels that are involved in a dizzying array of sensory mechanisms. Two channels in this large family TRPV5 and TRPV6 are highly Ca$^{2+}$ selective and are expressed in epithelia where they are important in Ca$^{2+}$ uptake. TRPV5/6 are constitutively active, yet the mechanisms regulating their activation in native tissue remains elusive. Here we functionally characterize the Xenopus TRPV6 homolog, xTRPV6 is expressed in the oocyte and encodes a channel that is permeant to divalents including Ca$^{2+}$, and displays a high permeability to Mg$^{2+}$. The oocyte does not exhibit functional TRPV6-like current at rest, showing that the endogenous channel is somehow maintained in an inactive state. We show that endogenous as well as overexpressed xTRPV6 interacts with xTRPC1 and that this interaction inhibits xTRPV6 currents. As such TRPC1 is likely to regulate the activity of TRPV6 under physiological conditions.

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Regulation of ionic homeostasis is essential for survival at both the organismal and cellular levels. The (TRP) gene family constitutes one of the major cation channel families and encodes tetrameric channels with the individual subunits composed of six membrane-spanning domains (TM), cytoplasmic N- and C- termini, and a pore region (P-loop) between TMS5 and TM6 (Wu et al., 2010; Nilius and Owsianik, 2011). This structure is reminiscent of voltage-gated K-channels and cyclic nucleotide-gated channels. The TRP gene superfamily is divided into seven families: TRPC (classical); TRPV (vanilloid); TRPM (melastatin); TRPP (polycystin); TRPML (mucolipin); TRPA (ankyrin); and TRPN (NOMPC). TRP channels are essential for many physiological processes, including Ca$^{2+}$ re-absorption in the kidney and intestine, sperm motility, detection of sensory inputs (pheromone, heat, cold), and smooth muscle contraction (Wu et al., 2010; Nilius and Owsianik, 2011). Most TRP channels are cation non-selective, and pass Ca$^{2+}$ into the cell down its electrochemical gradient. The exceptions are TRPV5/6, which are highly Ca$^{2+}$ selective ($P_{Ca}/P_{Na} > 100$) (Hoenderop et al., 2003b; Wissenbach and Niemeyer, 2007), and TRPM6/7 which form Mg$^{2+}$-permeant channels that are selective for transition metals, particularly Zn$^{2+}$ (Fleig and Penner, 2005; Bates-Withers et al., 2011; Runnels, 2011). The TRPC family encompasses seven members, which are most similar to Drosophila TRP, activated for the most part downstream of G-protein and tyrosine kinase-coupled receptors, that activate PLCβ, and PLCγ, respectively (Trebak et al., 2007). TRPC channels are mostly cation non-selective ($P_{Ca}/P_{Na}$ range: 1–9), and are broadly expressed (Trebak et al., 2007; Birnbaumer, 2009). The founding member of the TRPC family, TRPC1, has been implicated in a variety of cellular functions, including mechanotransduction (Maroto et al., 2005; Patel et al., 2010), growth cone guidance (Wang and Poo, 2005), the generation of excitatory postsynaptic potential (Kim et al., 2003) as well as neurological diseases such as Parkinson (Selvaraj et al., 2010). Furthermore, in the past decade, the contribution of TRPC1 to the store-operated Ca$^{2+}$ entry mechanism has been extensively studied and debated (Liu et al., 2003; Trebak et al., 2007; Cheng et al., 2011b).

The TRPV family has six members that exhibit an interesting range of gating mechanisms. For example, TRPV1 is activated by capsaicin (the active ingredient of chili pepper) and by heat (>42˚C), whereas TRPV4 is activated by osmolality and warm temperatures in a lower range (27–24˚C) (Wu et al., 2010). TRPVs can be further divided in two subfamilies: TRPV1-4, that are modestly Ca$^{2+}$ permeable with a $P_{Ca}/P_{Na}$ between 1 and 10 and TRPV5/6 that are highly Ca$^{2+}$-selective with a $P_{Ca}/P_{Na} > 10$ (Vennekens et al., 2001; Yue et al., 2001; Hoenderop et al., 2003b; Owsianik et al., 2006). TRPV5 is expressed primarily in the kidney and intestine, whereas TRPV6 is widely expressed (den Dekker et al., 2003; Owsianik et al., 2006; Wu et al., 2010). Currently the primary physiologically defined role of TRPV5/6 is Ca$^{2+}$ reabsorption in epithelia (Hoenderop and Bindels, 2008). The predicted tetrameric structure of TRP channels has been supported experimentally for numerous members of the super-family such as TRPC1 (Barrera et al., 2007), TRPV1 (Kedei et al., 2001; Zhang et al., 2011), TRPV5/6 (Hoenderop et al., 2003a; Niemeyer, 2005), TRPM6/M7 (Li et al., 2006), and TRPM8 (Stewart et al., 2010).

Abbreviations: TRP, transient receptor potential; TM, transmembrane domain; IC$_{50}$, Ca$^{2+}$-activated Cl$^{-}$ current; xTRPV6, Xenopus TRPV6; xTRPC1, Xenopus TRPC1.

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The subunit composition of the tetramer can be either homomeric or heteromeric. The formation of heteromeric channels was first thought to be mainly restricted to members of the same family or even to the same sub-family (Schafer, 2005) such as TRPC4/TRPC5 (Hofmann et al., 2002), TRPV1/TRPV2, TRPV5/TRPV6 (Heilwig et al., 2003), or TRPM6/TRPM7 (Chubanov et al., 2004). More recently, it has been shown that members from distinct families can also be involved in heteromeric channels such as TRPML3/TRPV5 (Guo et al., 2013) or TRPP2/TRPV4 (Kottgen et al., 2008). TRPC1 in particular has been shown to contribute to heteromers. First, TRPC1 can associate with several members of the TRPC family and the presence of TRPC1 tends to reduce the Ca\(^{2+}\)-permeability of those channels (Hofmann et al., 2002; Storch et al., 2012). Second, TRPC1 also associates with members of other families like TRPP2 or TRPV4, affecting in both cases the electrophysiological properties of the corresponding homomeric channel (Bai et al., 2008; Zhang et al., 2009; Ma et al., 2010, 2011b). The TRPV4/TRPC1 heteromeric channel has also been shown to contribute to store-operated Ca\(^{2+}\) entry in vascular endothelial cells (Ma et al., 2011a). Of relevance TRPC1 has been shown to be expressed in Xenopus oocyte (Bobanovic et al., 1999).

Here we show that the Xenopus TRPV6 homolog is expressed in Xenopus oocytes at high levels endogenously. However, no spontaneous TRPV6-like currents are observed in oocytes at rest. Overexpression of TRPV6 in the oocyte produces a Mg\(^{2+}\) and Ca\(^{2+}\) permeable channel. We also show that TRPV6 interacts with TRPC1 leading to inhibition of xTRPV6 function.

Methods
Ethical approval
Animals were handled according to Well Cornell Medical College IACUC approved procedures.

Molecular biology
Cloning of the Xenopus TRPV6 was accomplished by searching Xenopus EST databases, which identified a clone with high sequence identity to rat TRPV6. Using RT-PCR, and cDNA clones obtained from the Resource Center (RZPD, Germany) and the Xenopus EST project (NIBB, Japan), we obtained a full length cDNA for the Xenopus TRPV6 homolog and sequenced it on both strands (Fig. 1A). The sequence was deposited in GenBank with the accession number JX442252. For RT-PCR detection of TRPV6, the following specific primers were used: forward (CAGTGCTCACAGAAGCTCTCTTGGCGTCC) and reverse (CCATGGCGTACGCGATCCTGCGG). C-terminally tagged xTRPC1-GFP and xTRPV6-mCherry were constructed by first cloning GFP or mCherry between the dIII sites of pSGEM. xTRPC1 or xTRPV6 were then cloned in frame to the tags to generate the fusion proteins. The same approach was used to N-terminally tag xTRPC1 with GFP and xTRPV6 with mCherry. The clones for Orai-GFP and TMEM16A have been previously described (Yu et al., 2010, 2011).

Expression in Xenopus oocytes
Stage VI oocytes were harvested from wild type Xenopus laevis (Xenopus Express, France) using previously described procedures (Machaca and Hartzell, 1999). Typically, the cells were injected 24 h after harvesting with 20 ng of the RNA coding for TRP proteins and expression was allowed for at least 48 h. For Orai-GFP and TMEM16A, 2 and 8 ng of each coding mRNAs were injected, respectively.

Electrophysiology
The ionic currents were recorded using standard two electrode voltage-clamp recording technique. Recording electrodes were filled with 3 M KCl and coupled to a Geneclamp 500B controlled with pClamp 8.2 (Axon Instruments). Intracellular calcium levels were monitored using calcium-activated chloride currents as sensors as previously described (Machaca and Hartzell, 1999). The resting calcium concentration in the vicinity of the I_{Ca}\(_{\text{L}}\) channel is first recorded using a depolarizing voltage jump (-30 to +40 mV, I_{Ca}\(_{\text{L}}\)). A following hyperpolarizing pulse to -140 mV increases the driving force for calcium and allows calcium to flow through open plasma membrane channels, this leads to the recording of a mixed I_{Ca}\(_{\text{L}}\)/I_{Ca}\(_{\text{H}}\) current termed I_{Ca}\(_{\text{L}}\). The third voltage jump is identical to the first one, the size of the recorded current is an indicator of the calcium influx during the second pulse and is corrected from resting calcium levels using the I_{Ca}\(_{\text{L}}\) value (I_{Ca}\(_{\text{L}}\)T, Fig. 2A). To uncouple the activation of I_{Ca}\(_{\text{L}}\)/I_{Ca}\(_{\text{H}}\) channels from centrini the cells were injected with 250 mM BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N\(_{\text{,N}}\)\(_{\text{,N}}\)\(_{\text{,N}}\)-tetraacetic acid) to a final concentration of 2.5–5 mM.

Imaging
Confocal imaging of live cells was performed using a LSM710 (Zeiss, Germany) and a Plan Apo 63x/1.4 oil immersion objective or a TCS SP5 (Leica, Germany) fitted with a 63\(/\times1.4\)-0.6 oil immersion objective. The cells were maintained in normal saline (no perfusion) during the imaging process. z-stacks were performed in 0.5 \(\mu\)m sections using 1 Airy unit pinhole aperture. The images were recorded with Zen2008 (Zeiss) or LAS AF 2.4.1 (Leica) and analyzed with ImageJ 1.46q (Schneider et al., 2012).

Solutions
The cells were continuously superfused during voltage-clamp experiments using a peristaltic pump. The standard extracellular saline contained (in mM) 96NaCl, 2.5KCl, 1.8CaCl\(_{2}\), 2MgCl\(_{2}\), 10 HEPES, pH 7.4. For extracellular calcium manipulation the salines contained 40NaCl, 0.1–30CaCl\(_{2}\), and 15–63 Choline-Cl. For divalent permeability experiments the solution contained 90 Choline-Cl, 10× Cl\(_{2}\), 10 HEPES, pH 7.4. The correct osmolarity of the salines was measured using a Semi-Microsmometer (K7400, Knauer, Germany).

Immunoprecipitation
Cell lysates were prepared from 50 oocytes in each condition and centrifuged three times at 1,000g (4°C, 10 min), the supernatant was incubated with 4% NP-40 for 1 h before being centrifuged to 15,000g (4°C, 15 min). The lysates were then pre-cleared on agarose beads (20 l of the required agarose beads (20 l of the required agarose beads and incubated for 1 h. The mixture was centrifuged first at 3,000 rpm for 30 s at 4°C. The beads were then washed three times in IP lysis buffer and the supernatant discarded. Fifty microliters of 2× loading buffer was then added to the beads and boiled at 95°C for 3 min. After centrifugation the supernatant was frozen or loaded on a gel.

Surface biotinylation
Oocytes were incubated on ice for 30 min, washed three times on ice with PBS, then incubated with 0.5 mg/ml Sulfo-NHS-IC-Biotin (Pierce, Rockford, IL) in PBS, pH 8 at 4°C for 2 h with end
Fig. 1. TRPV6 and TRPC1 are expressed in *Xenopus* oocytes. A: Sequence alignment of rat TRPV5 and TRPV6 together with the *Xenopus* TRPV6 (xTRPV6). The ankyrin repeats (ANK1-4), membrane spanning domains (TM1-6) and the putative pore region (P-loop) are indicated. B: Phylogenetic analysis of xTRPV6 (JX442252) with human (h) and rat (r) members of the TRPV family: hTRPV1 (NP_542436.2), hTRPV2 (NP_057197.2), hTRPV3 (NP_659505.1), hTRPV4 (NP_067638.3), hTRPV5 (NP_062815.2), hTRPV6 (NP_061116.2), rTRPV6 (NP_446138.1). The tree was constructed using ClustalW and neighbor joining building method. C: Hydropathy plot suggesting the membrane topology of the xTRPV6 protein (Kyte–Doolittle method, sliding window of nine amino acids) using Expasy tools (Gasteiger et al., 2005), the putative transmembrane segments are numbered 1-6, P indicates the pore loop. D: Detection of xTRPV6 mRNA in naïve oocytes. Total RNA from *Xenopus* oocytes was isolated using standard techniques and amplified in a RT-PCR using specific xTRPV6 primers: forward (CAGTGCTCACAGAAGCTCCTCTTGGCGTCC) and reverse (CCAGTGCGTGTCAGCCATCATGCG). The negative control (Ctr) was a similar RT reaction without template RNA. E: Detection of the immuno-reactivity for TRPC1 and TRPV6 in naïve *Xenopus* oocyte lysates. The equivalent protein content of two cells per lane was analyzed on a 10% SDS–PAGE and transferred on a PDVF membrane. Both TRPV6 and TRPC1 are detected in the cell lysates. The TRPV6 protein is detected as a slightly lighter molecular weight than predicted (~70 kDa vs. 79 kDa). In our experimental conditions this is also the case for mammalian TRPV6 proteins (see Supplementary Fig. 1).
Fig. 2. Overexpression of xTRPV6 in Xenopus oocytes. A: The triple voltage jump protocol allows the detection of calcium influx through the plasma membrane. The current induced by the first depolarization to +40 mV (I_{Cl,Ca1}) measures Cl current at the resting Ca^{2+} or during Ca^{2+} release. The following hyperpolarizing pulse drives Ca^{2+} inside the cell and activates the chloride channels inducing I_{Cl,Ca2}. The third step is identical to the first one and measures the Cl current activated in response to Ca^{2+} influx induced by the hyperpolarization pulse. The value of the current is corrected for I_{Cl,Ca1} (background) and termed I_{Cl-T}. This current is strongly reduced by bath application of 200 μM La^{3+}. B: Bar chart summarizing the effects of La^{3+} and ruthenium red (Rut Red) on the Ca^{2+} influx recorded in cells overexpressing xTRPV6. C: The inactivation of the xTRPV6 current during a single voltage pulse to 140 mV is more pronounced when the extracellular calcium concentration is raised from 2 to 30 mM (extracellular Na^{+} is kept to 40 mM). D: Bar chart illustrating the Ca^{2+}-dependent inactivation of the xTRPV6 from the ratio of the current amplitude at the peak of the current to its value at the end of the voltage pulse. E: Family of La^{3+}-sensitive currents recorded from 140 to +40 mV in 20 mV increments after BAPTA injection (final concentration 2.5–5 mM in the cell) to block ClCa channels activation. The current traces are corrected from the leak currents remaining after application of 200 μM La^{3+}. F: Current–voltage relationship of the currents recorded using the protocol described in (E) revealing the inward rectification pattern. All recordings are performed in normal Ringer unless otherwise stated. Significance was tested using Student paired t-test.
to end shaking. Oocytes were washed four times then quenched with 100 mM glycine in PBS for 1 h at 4°C and then lysed in lysis buffer (150 mM NaCl, 20 mM Tris, 1% Triton X-100, pH 7.5), the yolk was removed by centrifugation at 1,000 g for 10 min. Supernatants were then incubated with immobilized NeutrAvidin beads (Thermo Scientific, Rockford, IL) for 1 h at 4°C. The beads were centrifuged, washed three times with PBS and biotinylated proteins were eluted from the beads using Laemml SDS–PAGE sample buffer at 65°C for 10 min. Both input cell lysates and biotinylated samples were subjected to SDS–PAGE and Western blot analysis.

Western blots

Samples were boiled in loading buffer at 95°C for 3 min and ran on a 10% SDS–PAGE. The proteins were then transferred on a PVDF membrane at 20 V for 30 min and the membrane saturated in TBST for 1 h. The membranes were incubated overnight (4°C) with the primary antibody and 1 h at room temperature with the secondary. Detection was achieved using Pierce ECL Western blot detection kit.

Antibodies and buffers

To detect xTRPC1 the mouse anti-TRPC1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA) (SC-133076) was used. For xTRPV6 we used the rabbit anti-TRPV6 antibody from Santa-Cruz (SC-28763, Fig. 1). For some experiments (Fig. 5) the Alomone Labs anti-TRPV6 antibody (ACC-036) was used, and it gave identical results to the Santa Cruz antibody. The Santa-Cruz TRPV6 antibody was tested on the human small intestine cell line (FHs74 Int) and on rat brain extracts (brain, Supplementary Fig. 1A). Antibodies were used at a 1:200 dilution for Western blots (TRP proteins). For loading controls the following antibodies were used: GAPDH (Santa Cruz sc-137179, 1:200), actin (Sigma–Aldrich, Saint Louis, MO; A1978, 1:10,000) and tubulin (Sigma T9026, 1:500). The lysis/immunoprecipitation buffer contains: 30 mM Hepes, 100 mM NaCl, protease inhibitor cocktail III (Calbiochem, Billerica, MA; A137179, 1:200), actin (Sigma–Aldrich, Saint Louis, MO; A1978, 1:200), and phosphatase inhibitor cocktail 2 and 3 (Sigma, 1:100).

Statistics

Values are given as means ± SEM. Statistical analysis was performed when required using either Student paired and unpaired t-test or ANOVA followed by Newman–Keuls post hoc test. P is indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ns (not significant). Statistics were obtained using Prism 5.04 (Graphpad Software, La Jolla, CA).

Results

Xenopus laevis oocytes express a protein homologous to mammalian TRPV5/6

A full length cDNA encoding the Xenopus TRPV6 homolog was obtained as described in Methods Section (Fig. 1A). The sequence was confirmed using several additional cDNA clones from the Xenopus EST project, and fragments from xTRPV6 obtained by RT-PCR using total oocyte RNA as a template. Sequence alignment of xTRPV6 with rat TRPV5 and TRPV6 reveals a high level of identity over the entire length of the protein (52% and 59%, respectively), with the C-terminal region being the least conserved (Fig. 1A). Phylogenetic analysis shows that xTRPV6 is most closely related to mammalian TRPV5 and TRPV6 proteins among other members of the TRP family (Fig. 1B). This is the only TRPV5/6 homolog identified in Xenopus laevis, and it shares 57% and 50% identity with the predicted TRPV6 (XP_002944196) and TRPV5 (NP_001186854) proteins of Xenopus tropicalis, respectively. We therefore refer to this protein as xTRPV6 although a TRPV5 candidate has not yet been identified in laevis. The predicted topology of xTRPV6 fits the TRP superfamily mold, with six membrane spanning domains (TM), a putative pore region (P-loop) between TM5 and 6, and four ankyrin repeats (ANK) in the N-terminal region (Fig. 1A). xTRPV6 transcript was readily detected in oocyte total RNA extracts using RT-PCR (Fig. 1D). xTRPV6 protein expression was evaluated in oocyte lysates, revealing a single immunoreactive band that runs slightly faster (~70 kDa) than the predicted molecular weight of xTRPV6 (79 kDa, Fig. 1E). An anti-TRPV6 immunoreactive band of similar molecular weight was detected in both the human intestinal cell line FHs74Int and rat brain extracts (Supplementary Fig. 1A). In parallel, we also confirmed previous reports showing that xTRPC1 is expressed in Xenopus oocytes (Bobanovic et al., 1999; Breeroton et al., 2000). Oocyte lysates were probed with an anti-TRPC1 antibody, revealing a single band of a molecular weight (~100 kDa) slightly higher than the predicted value (90 kDa) for the unglycosylated xTRPC1 protein (Fig. 1E).

Overexpression of xTRPV6 in Xenopus oocytes

In mammals, TRPV5/6 subunits are known to assemble into channels with a high permeability to Ca^{2+}. In Xenopus oocytes a Ca^{2+} -activated Cl⁻ current (I_{Cl,\text{Ca}}) at high density providing a readily recordable endogenous sensor for Ca^{2+} release and influx in the oocyte (Hartzell, 1996). In fact I_{Cl,\text{Ca}} is the predominant current in the oocyte. Importantly, the amplitude of I_{Cl,\text{Ca}} at depolarized potentials in excised inside-out patches responds linearly to physiological Ca^{2+} concentrations between 280 nM and 1.1 μM (Kuruma and Hartzell, 2000). Consequently, to evaluate the functional expression of xTRPV6 in the oocyte we used a triple voltage jump protocol (Fig. 2A) that allows us to monitor Ca^{2+} release from intracellular stores as well as Ca^{2+} influx through the plasma membrane (see Methods Section). The first depolarizing pulse allows the recording of I_{Cl,\text{Ca}} that measures steady state Ca^{2+} levels. The hyperpolarizing pulse induces a Ca^{2+} influx, this increases intracellular Ca^{2+} and enhances the activation of I_{Cl,\text{Ca}} during the final depolarizing pulse. Hence the amplitude of I_{Cl,\text{Ca}} during the first 40 mV pulse measures Ca^{2+} release from stores, while the difference between the second and first 40 mV pulses reflects Ca^{2+} influx. This value is referred to as I_{Cl,T} (for transient current). Indeed this has been confirmed directly using combined Ca^{2+} imaging and voltage recording (Machaca and Hartzell, 1999). Using this approach we could not measure any significant Ca^{2+} influx on naïve oocytes (Fig. 2A). The endogenous xTRPV6 protein expressed in the oocyte and detected by immunoblotting is therefore not active at resting conditions. To investigate its biophysical properties we therefore overexpressed xTRPV6. The oocytes were injected with 20 ng of RNA coding for xTRPV6 and allowed to express for at least 48 h. Oocytes overexpressing xTRPV6 displayed a large I_{Cl,T}, indicating that membrane hyperpolarization triggered Ca^{2+} influx (Fig. 2A) and showing that xTRPV6 is Ca^{2+} permeant. Although there are no specific inhibitors for TRPV5/6 channels, they have been reported to be sensitive to trivalent ions such as La^{3+} or Gd^{3+}, as well as ruthenium red (Clapham et al., 2005; Wissenbach and Niemeyer, 2007). Bath application of 200 μM La^{3+} successfully inhibited Ca^{2+} influx in cells expressing xTRPV6 (Fig. 2A,B), while ruthenium red (50 μM) only resulted in a small reduction of the current (Fig. 2B). This differentiates the pharmacology of the Xenopus channel from its vertebrate counterparts. In mammals, one feature of TRPV5/6 proteins is their regulation by extracellular calcium characterized by a calcium-dependent inactivation of the current during prolonged voltage steps.
This regulation could also be observed in cells overexpressing xTRPV6 using two different extracellular calcium concentrations and a constant 40 mM extracellular sodium concentration (Fig. 2C). The calculated ratio between the peak current amplitude and the value at the end of the pulse was significantly increased when the extracellular calcium concentration was raised from 2 to 30 mM (Fig. 2D). Although these data are consistent with the dependence of xTRPV6 inactivation on extracellular Ca$^{2+}$ concentration, one cannot rule out the possibility that some of this modulation is due to the regulation of I$_{Cl, Ca}$ given that it is both Ca$^{2+}$- and voltage-dependent (Kuruma and Hartzell, 2000).

A limitation of these protocols is that the activation of I$_{Cl, Ca}$ prevents the recording of pure cationic currents. We therefore injected the oocytes with BAPTA to un Couple the activation of I$_{Cl, Ca}$ from intracellular Ca$^{2+}$. The remaining current was then measured over a series of voltage steps from $-140$ to $+40$ mV. Bath application of La$^{3+}$ was then used to eliminate the xTRPV6 current. Figure 2E shows the family of traces that represent the La$^{3+}$-sensitive currents. The current–voltage relationship of the current amplitude at the end of each voltage pulse is plotted in Figure 2F and displays an inward rectification as previously reported for rat and human TRPV6 (Peng et al., 2000; Hoenderop et al., 2001).

**Permeability of the channel**

Another well-described characteristic of TRPV5/6 channels is the block of monovalent ion flow by divalent ions, known as the anomalous mole fraction effect (Owsianik et al., 2006; Wis- senbach and Niemeyer, 2007). To evaluate anomalous mole fraction behavior of xTRPV6 in the oocyte Ca$^{2+}$ influx was measured using I$_{Cl, Ca}$ (Fig. 3A), and the total current carried by xTRPV6 was recorded after BAPTA injection in Figure 3B. Both protocols were performed with extracellular Ca$^{2+}$ concentrations ranging from 0.1 to 30 mM and Na$^+$ was kept at 40 mM. The plot in Figure 3C illustrates the evolution of both parameters as a function of extracellular Ca$^{2+}$. With increasing extracellular Ca$^{2+}$, the total xTRPV6 current is strongly reduced while Ca$^{2+}$ influx increases (Fig. 3C).

In rat and human, the permeability of TRPV5/6 channels to different divalent ions is Ca$^{2+}$ > Sr$^{2+}$ > Ba$^{2+}$ > Mg$^{2+}$ (Peng et al., 1999, 2000; Yue et al., 2001; Wissenbach and Niemeyer, 2007). Since the sequence of the putative pore domain of xTRPV6 differs from that of its mammalian counterpart (see Figs. 1A and 8A), we measured the permeability of the Xenopus channel to various divalents. The current amplitude was measured using 10 mM Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, or Mg$^{2+}$ as sole charge carriers in naïve and xTRPV6 expressing oocytes injected with BAPTA (Fig. 4A,D). Unlike mammalian TRPV5/6, the permeability to Ca$^{2+}$, Sr$^{2+}$, Ba$^{2+}$, and Mg$^{2+}$ were similar (Fig. 4D). The slight increase observed with Sr$^{2+}$ is probably due to its low binding to BAPTA together with its pore dilation. More surprising was the very high permeability to Mg$^{2+}$ in xTRPV6 expressing oocytes (Mg$^{2+}$/Ca$^{2+}$ = 1.87 ± 0.13, n = 27, Fig. 4A, D). The rate of rise of the current was also significantly different between 10 mM Ca$^{2+}$ and 10 mM Mg$^{2+}$ (Fig. 4A). The Mg$^{2+}$ current amplitude increased during the voltage pulse, while the Ca$^{2+}$ current remained stable (~0.76 ± 0.2 nA ms$^{-1}$ vs. 0.01 ± 0.08 nA ms$^{-1}$, Fig. 4A). This effect is even more pronounced with increasing extracellular Mg$^{2+}$ concentrations (Supplementary Fig. 2). Therefore, in contrast to the current mediated by other divalents, which rapidly reached steady-state after the voltage pulse, the Mg$^{2+}$ current showed a time-dependent activation component (Fig. 4A). Even when the duration of the hyperpolarization voltage pulse was increased the Mg$^{2+}$ current was augmented concurrently (Supplementary Fig. 2A,B). Mammalian TRPV6 has been shown to be modulated by both intracellular and extracellular Mg$^{2+}$ (Voets et al., 2003). We were therefore interested in determining whether the slow time-dependent activation observed when Mg$^{2+}$ is the current carrier is an intrinsic property of xTRPV6, or whether it involves the activation of an oocyte endogenous current. We therefore injected large amounts of Mg$^{2+}$ (final concentration ~10 mM) directly into naïve oocytes and measured the resultant current either in normal Ringer or in the presence of high extracellular Ca$^{2+}$ (60 mM; Supplementary Fig. 2A,C). In both cases Mg$^{2+}$ injection was sufficient to activate an endogenous Mg$^{2+}$ influx pathway (Supplementary Fig. 2C), showing that the oocyte possesses a Mg$^{2+}$-activated Mg$^{2+}$ transport pathway that is likely activated by the Mg$^{2+}$ influx stimulated by TRPV6. We did not further characterize...
this endogenous Mg$^{2+}$ transport pathway, which to our knowledge has not been previously reported in the literature. In summary these results argue that xTRPV6 forms Mg$^{2+}$ permeable channels in contrast to its mammalian counterparts, and as such is likely to contribute to Mg$^{2+}$ homeostasis in the oocyte, which is tightly controlled (Gabriel and Gunzel, 2007).

Interactions between xTRPC1 and xTRPV6

TRPC1 is expressed in a broad array of cell types and is one of the most studied TRP channels to date (Wu et al., 2010). TRPC1 is also known to be expressed in the *Xenopus* oocyte and was thought to be involved in the formation of stretch-activated channels (Maroto et al., 2005), however this

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**Fig. 4.** Permeability of xTRPV6 and regulation by xTRPC1. A: The current elicited by a voltage jump to 140 mV after BAPTA injection was recorded using various extracellular divalent cations (10 mM) as single charge carriers, the osmolarity was compensated using choline-Cl. The currents have been recorded on naive cells (gray traces) and xTRPV6 expressing oocytes (black traces). There is a significant increase in the amplitude of the current recorded with 10 mM Mg$^{2+}$. B,C: Co-expression of xTRPC1 strongly reduced Ca$^{2+}$ influx induced by xTRPV6 expression alone (B) as well as xTRPV6 current (C). D: Bar chart summarizing the current (recorded after BAPTA injection) solely carried by various divalent cations (10 mM) in naive cells and in oocytes overexpressing xTRPV6 alone or together with xTRPC1. Co-expression of xTRPC1 inhibits xTRPV6 currents. Statistics inside groups were performed using ANOVA, Student unpaired t-test was used between two different conditions.
observation has not been substantiated. The real function of TRPC1 and whether it forms functional homomeric channels is still a matter of debate (Gottlieb et al., 2008). When xTRPC1 was overexpressed in *Xenopus* oocytes it did not result in any currents as compared with un.injected oocytes (Supplementary Fig. 3). This is consistent with previous reports showing intracellular localization of expressed TRPC1 (Beech et al., 2003). However, given that both TRPV6 and TRPC1 are expressed in the oocyte we wondered whether the two channels interact. To test this possibility we co-expressed xTRPC1 and xTRPV6, which surprisingly results in a drastic reduction in Ca\(^{2+}\) influx as reported by \(I_{\text{Ca}}\) (Fig. 4B); as well as a reduction in the xTRPV6-dependent ionic current in Ringer (Fig. 4C). This inhibition was apparent when different divalents (Ca\(^{2+}\), Mg\(^{2+}\), Ba\(^{2+}\), Sr\(^{2+}\)) were used as the charge carrier (Fig. 4D). To support further the hypothesis of a xTRPV6–xTRPC1 interaction we tested the ability of both proteins to co-immunoprecipitate in naive cells. Immunoprecipitation of xTRPV6 efficiently pulled down xTRPC1 and vice versa (Fig. 5A), indicating an interaction between the two proteins. B: The reverse experiments using anti-TRPC1 antibodies for the immunoprecipitation (IP) results in the pull down of xTRPV6, supporting the interaction between the two proteins. The Western blots were performed on cell lysates from one to two oocytes (Input) and immunoprecipitates from 50 oocytes (IP).

To independently verify the plasma membrane localization of xTRPV6 and TRPC1 we performed biochemical biotinylation experiments, where cell membrane proteins were biotinylated and isolated using NeutrAvidin beads as described in Methods Section. Consistent with the confocal imaging data, xTRPV6 and TRPC1 were enriched in the biotinylated fraction as compared to actin or GAPDH (Fig. 6D, naive, Supplementary Fig. 6). Importantly, this enrichment of xTRPV6 was also apparent in cells co-expressing xTRPC1 and xTRPV6, where biotinylated xTRPV6 was readily detectable with only trace amount of actin (Fig. 6D, TRPV6–TRPC1 injected, Supplementary Fig. 6). These data show that a subset of xTRPV6 traffics to the cell membrane both in naive cells and when it is co-expressed with xTRPC1. It also illustrates that both TRPC1 and TRPV6 are located at the plasma membrane in naive cells. To further analyze the localization of xTRPV6 and TRPC1 at the plasma membrane we expressed them with known membrane markers, the calcium channel Orai1-GFP (Yu et al., 2011) and the calcium-activated chloride channel TMEM16A (Yu et al., 2010). Co-expression of Orai-GFP and xTRPV6-Ch (Fig. 7A) confirmed that a percentage of the xTRPV6 protein localizes to the plasma membrane. This could be evaluated by the plot of the fluorescence over the z-stack that shows an important overlap between the two proteins (Fig. 7Ca). However in contrast to Orai1 which is enriched at the plasma membrane, a significant proportion of xTRPV6 localizes intracellularly (Fig. 7Ca). Co-expression of xTRPC1-GFP with TMEM16a shows primarily intracellular localization of xTRPC1 (Fig. 7B). This is apparent in the plot of the fluorescence intensity in Fig. 7B, which shows little overlap between the xTRPC1-GFP and TMEM16a-Ch fluorescence signals. For reference we also show the localization of STIM1-Ch and Orai-GFP after depletion of intracellular stores with TPEN (Fig. 7Cc). Under those conditions STIM1 and Orai colocalize in the same focal plane in these confocal stacks as previously described (Yu et al., 2009, 2010). However, although some membrane expression is present in the case of xTRPV6 and of the xTRPV6/xTRPC1, the majority of the TRPC1 and xTRPV6 proteins localize to intracellular compartments (Figs. 6 and 7). We have previously shown that Orai localizes to the recycling endosome in oocytes and recycles continuously between this compartment and the cell membrane (Yu et al., 2010). Intracellular xTRPV6 localizes to an intracellular compartment that is distinct from the Orai1-positive recycling endosome (Fig. 7A). We would speculate that xTRPV6 defines a more static endosomal compartment since endogenous xTRPV6 is maintained in an inactive state likely due to its intracellular localization.

**Pore mutation in the xTRPV6 channel**

In the case of mammalian TRPV5/6, a critical amino-acid in the channel pore is aspartate D\(^{542}\) (TRPV5 numbering) that induce strong reduction of xTRPV6-dependent \(I_{\text{Ca}}\) (Fig. 6A). Confocal imaging was performed using z-stacks across the plasma membrane of the oocyte and revealed variations in the expression pattern of TRP proteins. In cells overexpressing xTRPV6-Ch large intracellular patches of fluorescence were visible while a fainter but significant fluorescence was observed at the cell membrane (Fig. 6B). Conversely in the case of xTRPC1-GFP a reticular diffuse intracellular pattern was observed and no localization of the protein at the cell membrane plane (Fig. 6B). This pattern changed when both proteins were co-expressed. While xTRPV6-Ch had a similar localization to when it was expressed alone, the sub-cellular distribution of xTRPC1-GFP was altered where it co-localized with xTRPV6-Ch to intracellular dense structures as well as to the plasma membrane (Fig. 6C).
modulates the high calcium selectivity of the channel (Owsianik et al., 2006). Alignment of xTRPV6 with its mammalian homologs shows that this aspartate is replaced by an asparagine (Fig. 8A). This substitution might contribute to the lower Ca\(^{2+}\) permeability of the channel. We therefore mutated the corresponding asparagine in xTRPV6 to an aspartate (xTRPV6N525D), and characterized it. xTRPV6N525D supports calcium influx at levels similar to xTRPV6 (Fig. 8B). The current–voltage relationship obtained in the same conditions as in Figure 2 was identical to the wild type.
Fig. 8. Mutation in the putative pore domain of xTRPV6. A: Sequence alignment of the putative pore domain of the rat TRPV6 (rTRPV6), *Xenopus* (xTRPV6), and the xTRPV6 mutant xTRPV6N525D. Numbering is according to rTRPV6 sequence. The highlighted residue (D) is known to control the permeability of the channel in rTRPV6. B: Recording of ICl-T current following over-expression of xTRPV6N525D in *Xenopus* oocytes shows that the channel is permeant to Ca\(^{2+}\) ions. As for the wild type xTRPV6, Ca\(^{2+}\) influx through xTRPV6N525D is inhibited by La\(^{3+}\) ions (200 μM). C: Comparison of the current–voltage relationship of wild type xTRPV6 and xTRPV6N525D (normalized to the value at 140 mV). This shows that the behavior of the mutant channel does not differ from the wild type over the voltage range studied. D: Compared anomalous mole fractions behavior recorded in oocytes expressing wild type xTRPV6 (black symbols) and xTRPV6N525D (white symbols) also failed to reveal any significant difference between the two proteins in the evolution of either the calcium influx (circles) or the xTRPV6 current recorded after BAPTA injection (squares).

Fig. 7. Co-localization of xTRPV6 and xTRPC1 with membrane markers. A: The plasma membrane localized calcium channel, Oral1-GFP (2 ng of RNA injected) was co-expressed with xTRPV6-Ch, and its expression pattern used to identify the plasma membrane plane where xTRPV6 is also located. Although both channels are present at the plasma membrane, they do not show any obvious co-localization either intracellularly or at the membrane plane. B: The Ca\(^{2+}\)-gated chloride channel TMEM16A-Ch was used as another membrane marker (8 ng of RNA injected), and reveals that the main localization of xTRPC1-GFP is intracellular. C: Graphs showing the distribution of the normalized fluorescence of different proteins over a z-stack. The focal plane of maximum expression for Oral1-GFP was defined as the membrane plane and the fluorescence intensity normalized to this value. The difference in the distribution of both proteins is highlighted as a blackened area between the two curves. A large overlap can be seen between TRPV6-Ch and Oral1-GFP (a) while TMEM16A-Ch and TRPC1-GFP show a more separate distribution (b), the third panel (c) illustrates the vicinity between the calcium sensor STIM1 and Oral1 after store depletion with TPEN as an example of co-localization of two proteins.
xTRPV6 (Fig. 8B). Moreover, the analysis of the anomalous mole fraction effect shows a comparable behavior between both proteins (Fig. 8D). Together these results suggest that this residue is not crucial in controlling the selectivity of the channel.

Discussion

The highly Ca\(^{2+}\)-selective TRPV5/6 channels are considered to be epithelial channels expressed primarily in the kidney and intestine where they mediate Ca\(^{2+}\) absorption (Hoenderop and Bindels, 2008; Woudenberg-Vrenken et al., 2012). Here we clone the Xenopus TRPV5/6 homolog and show that it is expressed in oocytes. Interestingly, although the xTRPV6 is readily detected at both the protein and mRNA levels in the oocyte, and expressed at the plasma membrane, functionally TRPV6-like currents are absent. Only after overexpression of xTRPV6 do we observe currents that share some characteristics of the TRPV5/6 family. The xTRPV6 forms Mg\(^{2+}\)-permeant channels that are also permeant to other divalents including Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\). The permeability to Mg\(^{2+}\) is higher than that for Ca\(^{2+}\) and no difference in the permeability to Ca\(^{2+}\), Ba\(^{2+}\), and Sr\(^{2+}\) could be detected, at least based on current amplitudes when the only permeant ion was the divalent being tested. In contrast, mammalian TRPV5 and TRPV6 show a voltage-dependent Mg\(^{2+}\)-block (Voets et al., 2003), and a different divalent cation selectivity (Ca\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\)) (Vennekens et al., 2000; Wissenbach and Niemeyer, 2007). The Mg\(^{2+}\) permeability is interesting and argues for a potential role for xTRPV6 in regulating Mg\(^{2+}\) homeostasis in the oocyte and potentially other cell types in the frog.

An aspartic acid residue in the mammalian TRPV6 has been implicated in both Ca\(^{2+}\) selectivity and the Mg\(^{2+}\)-block (Nilius et al., 2001; Woudenberg-Vrenken et al., 2012). In xTRPV6 this residue is occupied by a asparagine instead of the aspartic acid, arguing that it may be involved in xTRPV6 Mg\(^{2+}\)-selectivity. However, this was not the case since mutating NS25 to an aspartic acid (xTRPV6NS25D) displayed similar permeation, selectivity, and anomalous mole fraction behavior to wild type xTRPV6. This argues that the selectivity and permeation of xTRPV6 are defined by other residues in the pore or that the xTRPV6NS25D co-assembles with endogenous xTRPV6, forming a channel with properties that are similar to the wild type xTRPV6.

Although xTRPV6 is expressed in the oocyte it is not functional. The channel when overexpressed, as is the case for other TRPV5/6 channels, is constitutively open. This argues that either the channel in the oocyte does not traffic to the cell membrane or that it is maintained in an inactive state through interaction with other proteins. When xTRPV6 is overexpressed it results in robust currents of large amplitude in the oocyte, yet the majority of the protein remains intracellular in an endosomal compartment that is close to the cell membrane. This is in fact reminiscent of the sub-cellular distribution of endogenous or overexpressed TRPV5/6 proteins in various cell types such as retinal pigment epithelium (Kennedy et al., 2010), HEK293 (Hellwig et al., 2005), HeLa, and kidney cells (van de Graaf et al., 2006a). Several intracellular proteins acting as regulators of the localization of TRPV5/6 in the plasma membrane have been described (Schoeber et al., 2007), such as the small GTPase annexin 2 complex (van de Graaf et al., 2003), Rab11a (van de Graaf et al., 2006a), and Ezrin (Hatanou et al., 2013). The glycosylation state of TRPV5 has also been shown to be a key factor regulating membrane stability of the channel (Chang et al., 2005; Schoeber et al., 2007). Several proteins also co-immunoprecipitate with TRPV5/6 and regulate channel activity such as BSPRY, Numb1, and Calbindin-D28K (Lambers et al., 2006; van de Graaf et al., 2006b; Schoeber et al., 2007; Kim et al., 2013).

In oocytes, both endogenous and overexpressed xTRPV6 can be detected at the cell membrane. Interestingly, co-expression of xTRPC1 greatly diminishes the xTRPV6 associated current without affecting xTRPV6 protein levels or trafficking to the cell membrane. Upon co-expression of xTRPC1 with xTRPV6 the intracellular distribution of xTRPC1 is altered to mirror that of xTRPV6, showing that the two expressed proteins interact. Furthermore, immunoprecipitation experiments performed on naïve oocytes show that endogenous xTRPC1 and xTRPV6 associate. These results argue that xTRPC1 associates with and inhibits xTRPV6 in native oocytes and when both proteins are co-expressed. When xTRPV6 is expressed alone it is able to support a current, presumably because over-expressed xTRPV6 saturates endogenous xTRPC1 leading to its expression in an active form at the plasma membrane. Given that in most cells investigated the majority of the TRPV6 protein localizes to intracellular compartments it brings into question whether association with TRPC1 represents a potential mechanism responsible for activating TRPV6. While this work was in its final stages Schindl et al. (2012) reported a similar association between TRPC1 and TRPV6. However, in contrast to our findings they conclude that TRPC1 trapp TRPV6 intracellularly thus inhibiting TRPV6 currents. Our results argue that interaction of the TRPC1 with TRPV6 results in inhibition of TRPV6 function, since the trafficking of xTRPV6 was not altered following xTRPC1 expression. Furthermore, in native oocytes, which do not exhibit TRPV6 current, both xTRPV6 and xTRPC1 are found at the plasma membrane. Species and cell type differences could account for the differential regulation of TRPV6 by TRPC1. Further experiments are therefore required to understand the mode of activation in Xenopus oocytes of TRPV6 and the exact nature of the interaction between xTRPV6 and xTRPC1.

The involvement of TRPC1 in modulating the function of other TRP family members is not unique to TRPV6 and has been reported for other channels. TRPC1 association with other TRP proteins such as TRPP2, TRPV4, and other TRPCs has also been shown to affect the permeability and/or selectivity of the channel (Bai et al., 2008; Ma et al., 2011b; Storch et al., 2012; Beech, 2013). Enhanced trafficking to the membrane of TRPC1 by the TRPC4 subunit has also been reported (Hofmann et al., 2002). Furthermore, TRPC1 trafficking has been reported to be affected by associated proteins, such as RhoA and Cav-1 (Ong and Ambudkar, 2011). Ca\(^{2+}\) entry through the Orai1 channel was also identified recently as an important signal controlling TRPC1 insertion in the plasma membrane (Cheng et al., 2011a). Finally, overexpressed TRPC1 has been suggested to localize to intracellular compartments due to the lack of auxiliary subunits required for its trafficking (Beech et al., 2003).

Given that xTRPV6 is a Mg\(^{2+}\)-selective channel and that its tight selectivity is regulated it is interesting to note that there has been several indications of a function of Mg\(^{2+}\) in oocyte maturation. Increasing extracellular Mg\(^{2+}\) concentration has been reported to modulate germinal vesicle breakdown (Merriam, 1971; Baltus et al., 1977). Injection of Mg\(^{2+}\) into the oocyte has also been shown to accelerate the maturation of the oocyte induced by progesterone (Bellè et al., 1986). Since xTRPV6 overexpression results in a constitutively open Mg\(^{2+}\)-permeant channel, its regulation may be important in the context of Mg\(^{2+}\) homeostasis during oocyte maturation. Interestingly in that context, xTRPV6 current is completely inhibited in fully mature eggs (Supplementary Fig. 7). This suggests that either the channel is rendered non-functional during maturation, or that the protein is internalized as has previously been shown for the JOURNAL OF CELLULAR PHYSIOLOGY
calcium channel Oral and for the plasma membrane ATPase (El Jouti et al., 2008; Yu et al., 2009). These data argue that xTRPV6 function is regulated during oocyte maturation. TRPV5 and TRPV6 are expressed in epithelia of the digestive tract and kidney and are critical for Ca\(^{2+}\) absorption. They are tightly regulated by vitamin D and Ca\(^{2+}\), yet the detailed mechanisms regulating their activation and function remain elusive (Hoenderop and Bindels, 2008; Kutuzova et al., 2008; Woudenberg-Vrenken et al., 2012). TRPC1-dependent regulation of the epithelial Ca\(^{2+}\) channels has such pathophysiologic potential as regulating the activation of this unique subset of TRPV channels. However, more work is needed to better define the mechanisms by which TRPC1 regulates TRPV6 function especially under physiological conditions in epithelial tissue where TRPV6 function has been shown to be critical, but also in tissues such as the ovary where TRPV6 is expressed yet its functional contribution to ion homeostasis remains ill defined.

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Fig. S1. Western blots for TRPV6 and TRPC1. A: Cell lysates from an intestinal mammalian cell line (FHC74Int) and from rat whole brain were probed for TRPV6 immunoreactivity and both revealed a band slightly lower than the 72 kDa molecular weight marker. The membrane was also probed for tubulin (Tub) as a loading control revealing a 55 kDa band. B: Whole lanes from the Western blots probed for TRPV6 and TRPC1 from Fig. 1E.

Fig. S2. Magnesium permeability in Xenopus oocytes. A: Currents were recorded during a voltage jump to −120 mV for 1 min using 60 mM Mg2+ as sole extracellular charge carrier or normal saline (Ringer). Recordings have been performed on naïve cells, cells overexpressing xTRPV6 and after injection of 9.6 nL of 1 M MgCl2 for a final intracellular concentration of ~10 mM Mg2+. The Mg2+ injection is performed after the first recording. Both expression of xTRPV6 and injection of Mg2+ enhanced the magnesium permeability of the Xenopus oocyte. B: Time course of the development of the Mg2+ current in naïve and xTRPV6 expressing cells during repeated voltage jumps to −120 mV. C: Recording on naïve cells of the ionic current induced by injection of MgCl2. The currents are recorded in Ringer or using 60 mM Mg2+ as charge carrier. All cells have been injected with BAPTA (2.5–5 mM final concentration) to prevent the activation of ICl,Ca. The currents are measured at the beginning of the voltage jump.

Fig. S3. Overexpression of xTRPC1 in Xenopus oocytes. A: Recording of ICl,T as a measure of Ca2+ influx following overexpression of xTRPV6 or xTRPC1. xTRPC1 failed to generate any Ca2+-permeable channel in our recording conditions. B: Evaluation of the relative permeability at −140 mV for 10 mM of different divalent ions as single extracellular charge carriers (indicated above the bars). Oocytes overexpressing xTRPC1 did not differ from naïve oocytes in their permeability to the divalent cations evaluated.

Fig. S4. Whole lanes from the Western blots illustrated in Fig. 5. Oocyte lysates were immunoprecipitated with anti-TRPV6 (A) or anti-TRPC1 (B). The immunoprecipitates (IP) and the controls (Input) were probed on Western blots using anti-TRPV6 and anti-TRPC1 antibodies. Both experiments indicate co-immunoprecipitation of TRPC1 and TRPV6 proteins from Xenopus oocytes lysates. Note a shift in both the TRPV6 and TRPC1 immunoreactive bands in these IP experiments, which was due to the prolonged incubation of the lysates at 4°C during the immunoprecipitation procedure as also it occurred in untreated lysates. We do not fully understand the reasons for this shift but it could be due to modifications to the protein that are irreversible on SDS–PAGE.

Fig. S5. Overexpression of C-terminal tagged xTRP proteins in Xenopus oocytes does not produce any currents. A: Overexpression of mCherry-tagged TRPV6 and GFP-tagged TRPC1 at the C-terminal end resulted in no significant Ca2+ influx measured as ICl,T. B: The fluorescence could be visualized in the intracellular compartment for both xTRPV6-Ch and xTRPC1-GFP but only xTRPV6-Ch could be detected at the membrane plane.

Fig. S6. Whole lanes from the Western blots illustrated in Fig. 6. Intact Xenopus oocytes were biotinylated to evaluate the presence of TRPV6 and TRPC1 at the plasma membrane. A: naïve cells express TRPV6 and TRPC1 proteins at the plasma membrane as revealed by the immunoreactivity for both proteins in the biotinylated protein fraction (biotin). B: High amounts of TRPV6 protein can be detected at the membrane (biotin vs. Input) in cells overexpressing xTRPC1 and xTRPV6. The membranes were also probed for GAPDH and actin as controls for cytoplasmic contaminants.

Fig. S7. xTRPV6 is down-regulated in the egg. A: Comparative traces of ICl,T activation in a Xenopus oocyte and an egg overexpressing xTRPV6. The Ca2+ influx is absent in the egg. B: Bar chart summarizing the ICl,T current recorded in oocytes and eggs. The xTRPV6 current is totally inhibited in the egg. The cells were treated with 5 μg/ml progesterone overnight and recorded 30 min to 1 h after germinal vesicle breakdown. Statistics are according to Student unpaired t-test.