TRPM7: Ca2+ signaling, actomyosin remodeling and metastasis

Visser, Daan

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Chapter 4

Orchestrated PLC activation controls PIP$_2$ levels and Ca$^{2+}$-dependent TRPM7 opening

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Michiel Langeslag*, Daan Visser*, Agathe Bacquin, Jeffrey Klarenbeek, Frank N. van Leeuwen and Kees Jalink

* Contributed equally

Abstract

TRPM7 (Transient Receptor Potential subfamily Melastatin, member 7) is a non-selective divalent cation channel fused to a C-terminal alpha-kinase domain which has been implicated in various cellular processes, ranging from Mg$^{2+}$ homeostasis and proliferation to cytoskeletal remodeling, migration and metastasis. TRPM7 is under control of G$\alpha$q-coupled receptors and has been demonstrated to mediate localized Ca$^{2+}$ signaling. The molecular mechanisms that govern TRPM7 opening under physiological conditions and the downstream signaling pathways, however, are poorly understood and the role of phospholipase C - phosphatidylinositol(4,5)bisphosphate (PLC-PIP$_2$) signaling has been debated. We show that activation of TRPM7 in intact cells proceeds by a novel and unique mechanism that involves sequential activation of two PLC isoforms. PLC$\beta$$_3$ mediates initial activation of TRPM7 through IP$_3$-mediated release of Ca$^{2+}$ from ER stores and activation of the Ca$^{2+}$-sensitive isoform PLC$\delta$$_1$. Channel opening then leads to Ca$^{2+}$ influx which serves to prolong PLC$\delta$$_1$ activation and PIP$_2$ hydrolysis and thereby keeps the channel open. Thus, TRPM7 and PLC$\delta$$_1$ function together to form a feedforward regulatory loop whereby sustained Ca$^{2+}$ influx causes the PIP$_2$ hydrolysis necessary to keep TRPM7 open.

Keywords Ca$^{2+}$; FRET imaging; PIP$_2$; PLC; TRPM7
Introduction

The recent discovery of the large and diverse family of Transient Receptor Potential (TRP) cation channels has revolutionized our understanding of Ca\(^{2+}\) signaling pathways in the cell. Numerous members of the TRP channel family have been implicated in all of the major sensory transduction processes (Clapham, 2003) as well as in pathological conditions ranging from cancer and hypertension to hypomagnesemia, kidney disease and mucolipidosis (Middelbeek et al., 2012; Schlingmann et al., 2002; Sun et al., 2000; Vennekens, 2011; Woudenberg-Vrenken et al., 2009; Yogi et al., 2011). Although TRP channels are activated by a diverse array of mechanisms, the phospholipase C (PLC) - phosphatidylinositol(4,5) bisphosphate (PtdIns(4,5)P\(_2\)) signaling axis appears to be a common regulator of TRP channel gating.

Ca\(^{2+}\) and PIP\(_2\) are intimately linked in cells and on TRP-family channels, these signals appear to converge. Cleavage of PIP\(_2\) by receptor-operated PLC isoforms produces the soluble messengers inositoltrisphosphate (IP\(_3\)) and diacylglycerol (DAG). Whereas IP\(_3\) rapidly signals to IP\(_3\)-receptors in the ER Ca\(^{2+}\) store to mediate Ca\(^{2+}\)-release into the cytosol, DAG may influence ion channels either directly (Hardie, 2007) or through protein kinase C (PKC) (Soboloff et al., 2007). The concomitant drop in PIP\(_2\) levels also regulates a number of Ca\(^{2+}\)-permeable channels in the plasma membrane and, thereby, Ca\(^{2+}\) influx into the cytosol (Lukacs et al., 2013; Rohacs, 2009; Rohacs and Nilius, 2007; Suh and Hille, 2005; Yudin et al., 2011). Vice versa, Ca\(^{2+}\) stimulates phosphoinositide turnover via Ca\(^{2+}\)-sensitive PLCs (Rhee, 2001) and for example also affects type II PI\(_4\)-kinases (Wei et al., 2002).

TRPM7 is a member of the Melastatin subfamily of TRP channels, which conducts Ca\(^{2+}\) and Mg\(^{2+}\), associates with PLC isoforms and is regulated by PIP\(_2\) (Langeslag et al., 2007; Runnels et al., 2002; Xie et al., 2011). The sequence of TRPM7 reveals six transmembrane domains and long cytosolic C- and N-termini that contain sites for regulation and cofactor binding. TRPM7 also houses an alpha-kinase within its C-terminus capable of phosphorylating annexin-1 (Dorovkov and Ryazanov, 2004) and myosin II isoforms (Clark et al., 2006; Clark et al., 2008a; Clark et al., 2008b). TRPM7 is ubiquitously expressed and both its overexpression and ablation can cause rapid cell death (Jin et al., 2008; Nadler et al., 2001; Penner and Fleig, 2007; Ryazanova et al., 2010; Schmitz et al., 2003). TRPM7 has been implicated in numerous physiological cell processes, including Mg\(^{2+}\) homeostasis (Schmitz et al., 2003), proliferation (Abed and Moreau, 2007), embryonic development (Jin et al., 2008; Jin et al., 2012; Liu et al., 2011; Ryazanova et al., 2010), mechanotransduction (Numata et al., 2007; Oancea et al., 2006; Wei et al., 2009), cytoskeletal remodeling and in migration (Chen et al., 2010; Clark et al., 2006; Middelbeek et al., 2012; Su et al., 2006; Su et al., 2011; Wei et al., 2009). It is also associated with pathological disorders such as neuronal cell death (Aarts et al., 2003; Sun et al., 2009), hypertension (Touyz, 2008), and cancer (Dhennin-Duthille et al.,
Nevertheless, much of the details of the underlying signaling remain unclear and the mode of gating of TRPM7 continues to be debated. While emerging evidence shows that TRPM7 is the focal point of several signaling cascades, including phosphoinositides, cAMP, reactive oxygen species and pH (Aarts et al., 2003; Chen et al., 2012; Chokshi et al., 2012; Gwanyanya et al., 2006; Kozak et al., 2005; Runnels et al., 2002; Takezawa et al., 2004; Yogi et al., 2013), here we focus on the PLC-PIP$_2$ signaling axis.

Consistent with many other ion channels in the plasma membrane, TRPM7 requires at least a minimal level of PIP$_2$ for it to function (Gwanyanya et al., 2006; Kim et al., 2005; Kozak et al., 2005; Langeslag et al., 2007; Macianskiene et al., 2008; Oh et al., 2012; Takezawa et al., 2004; Xie et al., 2011). However, depending on the experimental conditions, activation of PLC appears to be either inhibitory or stimulatory for TRPM7. In whole-cell patch clamp studies, Mg$^{2+}$ is typically omitted from the intracellular solution to evoke large outward rectifying currents (Nadler et al., 2001) that are blocked by activation of PLCs or when PIP$_2$ levels are lowered in other manners (Runnels et al., 2001; Runnels et al., 2002). In contrast, in intact cells at physiological [Mg$^{2+}$], TRPM7 currents were augmented by agonists to PLC-coupled GPCRs such as bradykinin (BK), as demonstrated by perforated-patch and Ca$^{2+}$-fluorometry experiments (Langeslag et al., 2007). Indeed, biological effects downstream of TRPM7 appear to be enhanced, rather than inhibited, by PLC-activating stimuli (Callera et al., 2009; Clark et al., 2006; Kim et al., 2005; Langeslag et al., 2007; Wei et al., 2009; Yogi et al., 2009). The activation mechanism of TRPM7 and the contribution of PLC(s) and PIP$_2$ herein, however, has remained largely elusive. Furthermore, the effect of Ca$^{2+}$ influx through the channel domain on PIP$_2$ levels has not been determined to date.

We, therefore, set out to further study TRPM7-mediated Ca$^{2+}$ signaling with a focus on regulation by the PLC-PIP$_2$ signaling axis. Our results show that activation of PLC$_\beta_3$ and the ensuing release of Ca$^{2+}$ from ER stores are essential to initiate TRPM7 activation. Subsequently, Ca$^{2+}$ influx through TRPM7 activates the Ca$^{2+}$-sensitive isoform PLC$_\delta_1$ which is required for a sustained phase of TRPM7 opening. PLC$_\delta_1$-induced PIP$_2$ breakdown appears essential to keep the channel in the conductive state. Collectively, our experiments suggest that a feedforward loop involving Ca$^{2+}$ influx, PLC$_\delta_1$ activation and PIP$_2$ hydrolysis controls sustained TRPM7 opening.

**Results**

**TRPM7 activation through PLC$\beta_3$**

In empty-vector control N1E-115 neuroblastoma cells (N1E-115/EV), PLC activating agonists evoke a transient Ca$^{2+}$ peak that originates from intracellular stores [Fig. 1A], sometimes followed by a minor phase of Ca$^{2+}$ influx that is barely resolved from the baseline. We
established stable N1E-115/TRPM7 cell lines that overexpress TRPM7 just ~3-fold over endogenous levels (Clark et al., 2006; Langeslag et al., 2007). In these cells, the initial Ca\(^{2+}\) peak is followed by a marked sustained phase of elevated Ca\(^{2+}\) levels that lasts for a few to many minutes before returning to baseline [Fig. 1B]. This sustained phase results from Ca\(^{2+}\) influx through TRPM7 channels as we have extensively documented before (see Langeslag et al., 2007 in particular the appendix); for example, it is absent after knockdown of TRPM7.

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**Figure 1 | PLC\(_{\beta3}\) activation causes sustained Ca\(^{2+}\) influx through TRPM7.** (A) In N1E-115 empty-vector control cells, stimulation with BK evokes a single, transient increase in [Ca\(^{2+}\)]\(_i\), as detected by Ca\(^{2+}\) ratiometry (N > 500). (B) In contrast to N1E-115/EV cells (gray trace; for reference), the initial Ca\(^{2+}\) peak is followed by a sustained phase of Ca\(^{2+}\) elevation in N1E-115/TRPM7 cells (black trace) (N > 500); for detailed quantification, see (Langeslag et al., 2007). See text for further details. (C) RNAi against TRPM7 abolishes sustained Ca\(^{2+}\) signaling in N1E-115/TRPM7 cells (black trace). N1E-115/TRPM7 cells, control (N=9; gray trace; for reference), peak 79.2 ± 3.4%, sustained 30.0 ± 4.2%; TRPM7 RNAi (N=6; black trace), peak 75.0 ± 3.0%, sustained 10.4 ± 6.4%. (D) 2-APB rapidly blocks TRPM7-mediated Ca\(^{2+}\) influx to levels below basal [Ca\(^{2+}\)]\(_i\) (N=32). (E) Expression of GPCR-operated PLC isoforms in N1E-115 cells. (F) Knockdown of PLC\(_{\beta3}\) (lower panel) attenuates BK-induced Ca\(^{2+}\) transients and blocks sustained Ca\(^{2+}\) influx in N1E-115/TRPM7 cells (gray trace). Control (N=16; black trace), peak 55.8 ± 3.8%, sustained 16.5 ± 2.5%; PLC\(_{\beta3}\) RNAi (N=9; gray trace), peak 21.6 ± 6.7%, sustained 2.3 ± 0.9%. Shown are typical examples.
by shRNA [Fig. 1C] or in nominally \( \text{Ca}^{2+} \) free medium (not shown) and it is rapidly blocked by the TRPM7 blocker 2-aminoethyl diphenylborinate (2-APB) [Fig. 1D].

To elucidate the responsible signaling mechanism we initially focused on G\( \alpha \)q-coupled PLC isoforms, i.e. the PLC\( \beta \) forms (Rhee, 2001). RT-PCR revealed that N1E-115 cells express PLC\( \beta \), and predominantly PLC\( \beta \_3 \) [Fig. 1E]. Indeed, knockdown of PLC\( \beta \_3 \) by shRNA [Fig. 1F, lower panel] suppressed the sustained influx of \( \text{Ca}^{2+} \) through TRPM7, but it also blocked the initial \( \text{Ca}^{2+} \) mobilization from internal stores in many experiments [Fig. 1F]. In contrast, knockdown of PLC\( \gamma \), which couples to tyrosine-kinase receptors, was without effect, in good agreement with the lack of TRPM7 activation by these receptors in N1E-115 cells (data not shown). These experiments therefore identify PLC\( \beta \_3 \) as a critical link between G\( \alpha \)q and sustained \( \text{Ca}^{2+} \) influx via TRPM7.

**\( \text{PIP}_2 \) controls TRPM7 opening downstream PLC\( \beta \_3 \).**

Each of the three intracellular signals generated by PLC, namely (i) the drop in membrane \( \text{PIP}_2 \) levels, and concomitant formation of (ii) membrane-delimited DAG and (iii) water-soluble \( \text{IP}_3 \), is known to affect ion channel gating. \( \text{PIP}_2 \) controls a variety of channels, including many TRP-family members, in a positive and negative manner (Rohacs and Nilius, 2007; Suh and Hille, 2005). DAG may influence channels either through PKC or directly (Rohacs and Nilius, 2007), whereas \( \text{IP}_3 \) can gate channels directly (Dellis et al., 2006) as well as through the ensuing \( \text{Ca}^{2+} \) increase (Dellis et al., 2006; Rohacs and Nilius, 2007). We therefore set out to investigate the involvement of each of these branches.

Activating PKC by pretreatment with either the water-soluble DAG analog OAG or phorbol esters did neither affect basal \( \text{Ca}^{2+} \) levels nor influence agonist-induced TRPM7 activation, ruling out a role for DAG/PKC [Fig. 2A]. In contrast, raising intracellular \( \text{IP}_3 \) by UV-induced release of an inactive precursor (“caged \( \text{IP}_3 \” \); Li et al., 1998) consistently evoked both an initial peak and the sustained phase in N1E-115/TRPM7 cells [Fig. 2B, left panel]. Again, administration of \( \text{Ca}^{2+} \) chelators such as BAPTA or the channel blocker 2-APB terminated the sustained phase (data not shown). In control N1E-115 cells, uncaging of \( \text{IP}_3 \) caused transient \( \text{Ca}^{2+} \) release from internal stores, with no sign of sustained influx [Fig. 2B, right panel]. When the \( \text{Ca}^{2+} \) stores in N1E-115/TRPM7 cells were emptied by pretreatment with thapsigargin, subsequent stimulation with BK [Fig. 2C] failed to activate \( \text{Ca}^{2+} \) influx. Thus, \( \text{IP}_3 \) does not directly affect TRPM7 gating, but \( \text{IP}_3 \)-mediated \( \text{Ca}^{2+} \) signals suffice to trigger TRPM7 under these conditions.

To investigate to what extent an agonist-induced drop in \( \text{PIP}_2 \) levels is involved, we clamped \( \text{PIP}_2 \) levels by overexpressing P(4)P 5-kinase 1\( \alpha \) (PIPkin 1\( \alpha \)), the major \( \text{PIP}_2 \)-synthesising enzyme in these cells. This abolishes or severely blunts the agonist-induced drop in membrane \( \text{PIP}_2 \) without affecting the initial \( \text{Ca}^{2+} \) peak (van Zeijl et al., 2007) or
Figure 2 | The contribution of second messengers downstream PLCβ3 to TRPM7-mediated Ca²⁺ influx. (A) Administration of the water-soluble DAG analog OAG (left panel) or the phorbol ester PMA (right panel) neither mimic BK in activating TRPM7, nor affect the response to BK (N > 10). (B) Photorelease of caged IP₃ causes sustained Ca²⁺ influx in N1E-115/TRPM7 cells; (N=58) peak 2.25 ± 0.06 and sustained 1.24 ± 0.01. In contrast, in N1E-115/EV cells, very little sustained Ca²⁺ influx was evoked by uncaging IP₃; (N=31; trace not shown) peak 2.21 ± 0.10, sustained 1.03 ± 0.02. *, p << 0.00001. Quantification is shown in the right panel. (C) Thapsigargin pretreatment blocks both the initial Ca²⁺ peak and sustained Ca²⁺ influx through TRPM7. Cells were pretreated with 1 µM thapsigargin and Ca²⁺ signals were assayed by ratiometric imaging (N=11). (D) PIPkin 1α overexpression does not affect BK-induced generation of IP₃. IP₃ generation in N1E-115/TRPM7 control cells (black trace) and PIPkin 1α overexpressing cells (gray trace) were detected using an IP₃ FRET sensor. (E) Clamping PIP₂ levels prevents TRPM7 activation. Overexpression of PIPkin 1α blocks the BK-induced drop in PIP₂ levels (see lower panel photomicrographs). Under these conditions, BK fails to activate TRPM7 (gray trace). Cells were cotransfected with untagged PIPkin 1α and Yellow Cameleon 2.1, which served both as transfection marker and as read-out for Ca²⁺. N1E-115/TRPM7 control cells (N=13; black trace), peak 81 ± 5%, sustained 36 ± 5%; PIPkin 1α overexpressing cells (N=10; gray trace), peak 72 ± 7%, sustained 11 ± 5%. Typical experiments are shown. ‘b’, basal; ‘p’, peak; ‘s’, sustained phase.
BK-induced formation of IP$_3$ [Fig. 2D]. In all cases, overexpression of PIPkin 1α completely blocked TRPM7-mediated Ca$^{2+}$ influx in N1E-115/TRPM7 cells [Fig. 2E]; n = 10. These data demonstrate first, that a drop in PIP$_2$ levels is prerequisite for the activation of TRPM7, and second, that the production of IP$_3$, per se, is not sufficient to cause its sustained opening. In view of the well-documented dependency of whole-cell TRPM7 currents on the presence of (at least) a low level of PIP$_2$ at the membrane, the data also indicate that TRPM7 channel opening displays a biphasic dependency on PIP$_2$ levels. We refer to the supplemental material for additional experiments underpinning this dual regulation.

**Positive feedback controls sustained TRPM7 opening.**

As TRPM7 conductance thus critically depends on PIP$_2$ levels, we next used FRET (Fluorescence Resonance Energy Transfer) assays to study PIP$_2$ levels in N1E-115/TRPM7 and N1E-115/EV control cells under various conditions (Langeslag et al., 2007; van der Wal et al., 2001). In the latter, agonists such as BK evoke transient PIP$_2$ breakdown which recovers to baseline within 1-2 minutes [Fig. 3A, gray trace]. In N1E-115/TRPM7 cells, onset and peak of PIP$_2$ hydrolysis were very similar, but a distinct prolonged recovery phase was observed [Fig. 3A, black trace; for quantification see Fig. 3C and the legend]. Thus, PIP$_2$ hydrolysis mirrors agonist-induced Ca$^{2+}$ changes.

To test whether the prolonged phase of PIP$_2$ hydrolysis is dependent on Ca$^{2+}$ influx, we repeated these experiments in nominally Ca$^{2+}$-free medium. Strikingly, under these conditions the kinetics of PIP$_2$ hydrolysis in N1E-115/TRPM7 cells reverted to control [Fig. 3B, black trace]. A similar effect was observed when TRPM7 was blocked using for example 2-APB in normal Ca$^{2+}$-containing medium (data not shown). These data indicate that the sustained, but not the initial, phase of PIP$_2$ hydrolysis is significantly influenced by Ca$^{2+}$ influx. They also suggest that a remarkable feedforward regulatory loop is in place whereby Ca$^{2+}$ influx through TRPM7 controls sustained PIP$_2$ degradation, which in turn further augments TRPM7-mediated Ca$^{2+}$ influx.

A characteristic feature of feedforward mechanisms is that abrogating one of the components in the chain subsequently shuts off all events in the chain. If true, then briefly interrupting Ca$^{2+}$ influx should suffice to break the loop and shut down TRPM7. To test this, following recording of a baseline, TRPM7 was activated with BK and onset of the sustained Ca$^{2+}$ phase was monitored [Fig. 3D, left panel]. Using a perfusion pipette the cell was next briefly pulsed with nominally Ca$^{2+}$-free buffer (containing ~150 nM free Ca$^{2+}$ and 1 µM BK). This resulted in an immediate drop of cytosolic Ca$^{2+}$ levels [Fig. 3D, middle panel] that proceeded to values below (81 ± 1.7 %) baseline. This is in agreement with the notion
that TRPM7 contributes to the setpoint of basal Ca\(^{2+}\) levels in unstimulated cells (Aarts et al., 2003; Langeslag et al., 2007). As predicted, switching back to normal, Ca\(^{2+}\)-containing medium invariably caused intracellular Ca\(^{2+}\) levels to return to base line, rather than to the original open channel level (n = 23). Often, pulsing cells with nominally Ca\(^{2+}\)-free solution for just a few seconds sufficed to break the activation loop. This observation indicates that sustained Ca\(^{2+}\) influx is essential to keep TRPM7 in its open state.

**Figure 3 | TRPM7-mediated Ca\(^{2+}\) influx affects kinetics of PIP\(_2\) hydrolysis.** (A) Average BK-induced transient decrease in PIP\(_2\) levels in N1E-115/EV and N1E-115/TRPM7 cells, as detected by the FRET assay (see Materials and Methods). In N1E-115/EV cells (gray trace) FRET dropped by 79.9 \(\pm\) 6.0%, peaking at 20.4 \(\pm\) 1.4 s (N = 10). Recovery to within 87.5 % \((t_{7/8})\) of the peak response was in 92.1 \(\pm\) 11.3 s. In contrast, N1E-115/TRPM7 cells (black trace) display a distinctly prolonged phase of PIP\(_2\) hydrolysis. FRET loss was 76.3 \(\pm\) 6.7% peaking at 22.4 \(\pm\) 1.4 s; \(t_{7/8}\) was 225.8 \(\pm\) 22.9 s (N = 15). (B) When assayed in nominally Ca\(^{2+}\) free saline (gray trace), the PIP\(_2\) response in N1E-115/TRPM7 cells reverts to that of maternal control cells. FRET loss is maximally 76.5 \(\pm\) 9.1% at 20.7 \(\pm\) 1.4 s; \(t_{7/8}\) was 112.0 \(\pm\) 11.6 s (N = 7). Black trace, response of N1E-115/TRPM7 cells in Ca\(^{2+}\)-containing medium (as in A) for reference. Traces shown are average responses. (C) Comparison of data shown in A and B. *, p < 0.001 (D) Ca\(^{2+}\) levels recorded from N1E-115/TRPM7 cells during perfusion with various solutions as indicated. Left panel, switching to BK-containing solution evokes sustained Ca\(^{2+}\) elevation. Right panel, briefly switching to nominally Ca\(^{2+}\)-free solution aborts the sustained phase. See text for further details. Shown is a typical example of N = 23 experiments. (E) Quantification of experiments in D. *, p < 0.001 versus basal.
PLCδ couples Ca\(^{2+}\) influx to TRPM7 opening.

One component of the regulatory loop is still missing: an enzyme that couples Ca\(^{2+}\) entry to sustained PIP\(_2\) degradation. Within the PLC superfamily, several PLC subfamilies are activated by Ca\(^{2+}\), namely PLCδ, PLCζ and PLCζ. A RT-PCR revealed that N1E-115/EV (not shown) and N1E-115/TRPM7 cells express PLCδ\(_1\), PLCδ\(_3\) and PLCη\(_1\) [Fig. 4A, upper panel]. We designed specific short-hairpin RNAs against these PLC isoforms and knockdown was verified by semi-

Figure 4 | PLCδ\(_1\) couples Ca\(^{2+}\) influx to sustained TRPM7 opening. (A) Endogenous expression of PLC isoforms in N1E-115 cells. Upper panel, RT-PCR shows expression of GPCR effector PLCβ\(_3\) and Ca\(^{2+}\)-sensitive PLCδ\(_1\), PLCδ\(_3\) and PLCη\(_1\). Lower panels, efficacy of shRNAi vectors for PLCδ\(_1\) and PLCη\(_1\). (B) RNAi against PLCδ\(_1\) abolishes sustained Ca\(^{2+}\) influx in N1E-115/TRPM7 cells. Knock-down of PLCδ\(_1\) attenuates sustained Ca\(^{2+}\) influx in N1E-115 TRPM7 cells (red trace) whereas PLCη\(_1\) knockdown has no effect (gray trace) compared to untransfected controls (black trace). Lower panel shows a blow-up of the sustained phase. Right panel shows quantification at the peak and sustained phase for untransfected N1E-115/TRPM7 cells (N = 24) and for cells transfected with shRNA against PLCδ\(_1\) (N = 22) or against PLCη\(_1\) (N = 10); *, p < 0.001. Ca\(^{2+}\) was read out using Yellow Cameleon which also served as transfection marker. (C) Overexpression of PLCδ\(_1\) augments BK-induced sustained Ca\(^{2+}\) influx through TRPM7 (green trace). N1E-115/TRPM7 cells were transiently transfected with GFP-PLCδ\(_1\) and assayed for Ca\(^{2+}\) by Fura-Red recording (lower panel photomicrographs). Final calibration was by ionomycin and excess Ca\(^{2+}\). *, untransfected cells. Typical experiment of N = 14. (D) PLCδ\(_1\) is enriched in invadosomes. GFP-PLCδ\(_1\) (green) colocalizes with the actin-dense core (red) of invadosomes visualized with Alexa-568-phalloidin (see also line profiles).
quantitative PCR [Fig. 4A, lower panel; Table S1; Table S2]. Individual shRNA constructs were cotransfected in N1E-115/TRPM7 cells together with the (genetically encoded) Ca$^{2+}$ sensor Yellow Cameleon. Strikingly, when cells were challenged with BK, PLC$\delta_1$ knockdown strongly suppressed sustained Ca$^{2+}$ influx [Fig. 4B]. In contrast, knockdown of PLC$\eta_1$ or PLC$\delta_3$ (data not shown) did not affect the response.

If knockdown of PLC$\delta_1$ abolishes TRPM7-induced Ca$^{2+}$ influx, then overexpression of the enzyme is expected to augment sustained influx in N1E-115/TRPM7 cells. Cells were transiently transfected with GFP-tagged PLC$\delta_1$ and loaded with the Ca$^{2+}$ dye FuraRed. This allows simultaneous monitoring of Ca$^{2+}$ kinetics in PLC$\delta_1$ overexpressing (GFP-positive) and neighboring untransfected cells (GFP-negative) [see Fig. 4C, lower panel]. Indeed, in GFP-PLC$\delta_1$ positive cells stimulation with BK evoked a much larger sustained phase of Ca$^{2+}$ influx. Again, this phase was completely blocked by buffering of Ca$^{2+}$ with excess BAPTA (not shown) or by 2-APB [Fig. 4C].

The relatively high levels of Ca$^{2+}$ necessary for full activation of PLC$\delta_1$ (in the micromolar range, Allen et al., 1997) are not generally reached throughout the cytosol during normal Ca$^{2+}$ signaling, but are documented to exist close to the pore of Ca$^{2+}$ channels, including IP$_3$-receptor channels (Patterson et al., 1999) and therefore, possibly, also TRPM7 channels (Wei et al., 2009). However, whereas PLC$\gamma_1$ and several PLC$\beta$ isoforms were found to weakly interact with the TRPM7 kinase domain in glutathione S-transferase (GST) pulldown purification assays, there are conflicting results for the interaction with PLC$\delta_1$ (Runnels et al., 2002; Xie et al., 2011). Alternatively, we therefore studied the localization of PLC$\delta_1$ with respect to TRPM7 by confocal imaging of GFP-PLC$\delta_1$. Full-length PLC$\delta_1$ was found localized predominantly at the plasma membrane, where it is known to bind with its PIP$_2$- and PS binding domains (Lemmon and Ferguson, 2000; Paterson et al., 1995). We also found PLC$\delta_1$ enriched in invadosomes [Fig. 4D], in a subset of N1E-115/TRPM7 cells, where we previously detected TRPM7 by immunolocalization (Clark et al., 2006 and Chapter 5). This shows that the enzyme may localize in close proximity to TRPM7 channels. Collectively, these data indicate that PLC$\delta_1$ is the Ca$^{2+}$-sensitive phosphoinositidase operating within the TRPM7 regulatory loop.

**Discussion**

The model presented in Figure 5 holds that activation of G$\alpha_q$-coupled receptors activates PLC$\beta_3$ [Fig. 1F] to generate second messengers that trigger the release of Ca$^{2+}$ from internal stores [Fig. 2C]. Locally, Ca$^{2+}$ levels raise high enough to activate PLC$\delta_1$ [Fig. 3; Fig 4], which is present together with TRPM7 at the plasma membrane [Fig. 4]. In turn, PLC$\delta_1$ degrades PIP$_{2}$, which we propose to be necessary for sustained TRPM7 opening [Fig. 3]. Thus, Ca$^{2+}$ entering through the channels prolongs the activation of PLC$\delta_1$ and thereby, activity of
TRPM7, effectively constituting a positive feedback (or feedforward) loop. Interestingly, this activation loop is also self-limiting, that is, it contains negative feedback as well. In particular, activation limits itself because (local) near-complete depletion of PIP$_2$ does not support TRPM7 conductance [Fig. S1], in accordance with previous reports (Langeslag et al., 2007; Macianskiene et al., 2008; Runnels et al., 2002; Xie et al., 2011).

Whereas our model describes activation and maintenance of the conductive state, it does not address termination. A feedforward regulatory loop can be terminated by

![Diagram of receptor-operated feedforward regulation of Ca$^{2+}$ entry through TRPM7.](image)

**Figure 5 | Model of receptor-operated feedforward regulation of Ca$^{2+}$ entry through TRPM7.** (1) Initial TRPM7 activation (black arrows): Following activation of PLC$\beta_3$ by Gq-coupled receptors, PIP$_2$ is hydrolyzed, forming IP$_3$ (and DAG), and Ca$^{2+}$ is released into the cytosol by IP$_3$ receptor channels present in the ER-membrane. This causes Ca$^{2+}$ levels to (locally) rise high enough to activate PLC$\delta_1$, which localizes in close to proximity of TRPM7 channels. The PLC$\delta_1$-mediated drop in PIP$_2$ facilitates the opening of TRPM7 channels and subsequent Ca$^{2+}$ influx. (2) Sustained TRPM7 opening (dashed gray arrows): Ca$^{2+}$ influx through TRPM7 (locally) promotes prolonged activation of PLC$\delta_1$ and PIP$_2$ breakdown, and thereby sustains TRPM7 opening. (?) Biological response to sustained TRPM7 opening: Continued Ca$^{2+}$-influx through TRPM7 may facilitate the activation of downstream effectors and signaling pathways: the association of the TRPM7 kinase domain with myosin II, for example (solid gray arrow), which would trigger myosin II heavy chain phosphorylation and actomyosin remodeling (Clark et al., 2006; Clark et al., 2008a; Clark et al., 2008b).
breaking it at any stage. Conceivably, termination could be by inactivation or dislocation (e.g. internalization) of TRPM7 and PLC6γ, or when PIP₂ runs out. The latter would trigger a reverse-avalanche effect whereby gradual closure of TRPM7 would diminish Ca^{2+} influx, leading to further inactivation of PLC6γ, and so on. In line with this reasoning, we find that the sustained Ca^{2+} phase most often displays a relatively stable plateau phase, which returns to base line rather abruptly after a variable time span [Fig. 1] (Langeslag et al., 2007).

TRPM7 has been shown to be an essential determinant of spatially constricted Ca^{2+} signals (or Ca^{2+} sparks/flickers) that have been proposed to guide cell migration (Wei et al., 2009). Furthermore, many of the regulatory components of the actomyosin cytoskeleton bind PIP₂. It is therefore tempting to speculate that the here described regulatory loop involving TRPM7 would locally modulate the actomyosin cytoskeleton, in particular since the association of its kinase domain with the myosin heavy chain is Ca^{2+}-dependent (Clark et al., 2006; Clark et al., 2008a). In Chapter 5, we addressed this hypothesis in detail. Remarkably, in that analysis we find no evidence that TRPM7-mediated local Ca^{2+} sparks, or, for that matter, global Ca^{2+} fluxes control invadosome dynamics in N1E-115/TRPM7 cells.

We also addressed the paradoxical results that appeared in literature on PIP₂ dependency of TRPM7 gating. The data described here and available in the literature (e.g. Langeslag et al., 2007; Runnels et al., 2002) indicate that moderate PIP₂ breakdown such as caused by activated (endogenous) receptors, stimulates the channels and is in fact essential, as evidenced by the inhibitory effect of clamping PIP₂ levels by PIP kinase 1α overexpression [Fig. 2D & E]. On the other hand, near-complete wipe-out of the PIP₂ pool, for example by rapamycin-induced recruitment of IPP to the membrane [Fig. S1], completely blocked TRPM7-mediated Ca^{2+} influx, in line with whole-cell patch clamp data. This indicates a biphasic dependency of the channel on PIP₂. Interestingly, a similar biphasic dependency on PIP2 levels has been proposed for TRPM8 (Rohacs et al., 2005; Yudin et al., 2011) and for TRPV1 (Lukacs et al., 2007; Lukacs et al., 2013).

Dual regulation by PIP₂ suggests the existence of more than one PIP₂-sensing motive within the TRPM7 sequence. Interestingly, the C-terminus of TRPM7 houses several potential PIP₂-interacting sites, namely the TRP domain (Rohacs et al., 2005; Xie et al., 2011), and two further polybasic stretches which we termed P1 (aa 1147-1154) and P2 (aa 1196-1218); the latter one homologous to a PIP₂ binding site in TRPV1 (Prescott and Julius, 2003). Three conserved basic residues within the TRP domain (positively charged residues K1112, R1115 and K1125) that were proposed to bind PIP₂ are required for proper channel activity of TRPM7, as well as in TRPM6 and TRPM8 (Rohacs et al., 2005; Xie et al., 2011). However, to obtain direct evidence of binding of PIP₂ to these sites remains a challenge. We have also identified a so-called split PH domain (van Rossum et al., 2005) within the C-terminus of TRPM7 (M. Langeslag and K. Jalink, unpublished observations). This domain,
located between aa 1643-1658 within the kinase domain is predicted to combine with a complementary halve present within PLCγ1 to form a complete PH domain with PIP2-binding properties. Interestingly, PLCγ1 interacts with the TRPM7 kinase-domain in GST pulldown purification assays (Runnels et al., 2002). On the other hand, in our experiments PLCγ1 knockdown did not noticeably affect TRPM7 activation in N1E-115 cells, indicating that the split PH domain or the interaction with PLCγ1 has little effect on the regulation of TRPM7. It is also conceivable that additional phosphoinositide-dependent mechanisms play a role in biphasic regulation of TRPM7. For example, ion channels may be controlled by pinch-off from- or fusion with the plasma membrane of TRPM7-containing vesicles, processes with documented PIP2/PIP3 dependency (Oancea et al., 2006; Yaradanakul et al., 2007). Finally, PIP2 sensitivity could reside in one of the many proteins that interact with TRPM7 (see Chapter 3) as was, for example, described for A-Kinase Anchoring Protein 150 and TRPV1 sensitivity to PIP2 (Jeske et al., 2011). This latter option would explain why the dual regulation was thus far not detected in whole-cell patch clamp studies, in which the channels are typically highly overexpressed and interactors may thus be lacking. Like in other TRP-family channels (Rohacs, 2009), the regulation of TRPM7 by membrane phosphoinositides thus appears highly complex and further experimentation is needed to resolve this issue. Similarly, other details of the regulatory loop described in this chapter await further study. It is, for example, not well understood how termination of Ca2+ influx is orchestrated.

In conclusion, we here described, for the first time, components of a feedforward regulatory loop that controls activation of TRPM7. We conclude that PLC isoforms and PIP2 hydrolysis are required to explain the time course of Ca2+ influx through the TRPM7 channel, and vice versa, that TRPM7 controls PLC activation and PIP2 levels. Our experiments thus provide a good starting point for further elucidation of TRPM7-mediated Ca2+ signaling in cellular processes.

Materials and methods

Materials

Ionomycin, bradykinin and thapsigargin were from Calbiochem-Novabiochem (La Jolla, CA, USA). 2-Aminoethyl diphenylborinate (2-APB), 1-Oleoyl-2-acetyl-sn-glycerol (OAG), Phorbol 12-myristate 13-acetate (PMA), wortmannin and rapamycin were from Sigma-Aldrich (St. Louis, MO, USA). Fura Red-AM, Oregon Green 488 BAPTA-AM, EDTA-AM and Phalloidin-Alexa-586 were from Invitrogen-Molecular Probes (Eugene, OR, USA). ISO-Ins(1,4,5)P3/PM (caged) was from Alexis Biochemicals (Lausen, Switzerland). Salts were from Merck (Darmstadt, Germany). Dulbecco’s MEM, fetal calf serum, penicillin and streptomycin were obtained from Gibco BRL-Invitrogen (Paisley, Scotland). FuGene HD transfection reagent was from Roche Diagnostics B.V. (Penzbreg, Germany). PLCβ antibodies were from Santa Cruz Biotechnology (Santa Cruz, USA) and the TRPM7 antibody from Alomone Labs (Jerusalem, Israel). HRP-conjugated secondary antibodies were from DAKO (Glostrup, Denmark).
Constructs
eGFP-PH(PLC\(\delta_1\)), eCFP-PH(PLC\(\delta_1\)) and eYFP-PH(PLC\(\delta_1\)) in pcDNA3 vectors are as previously described (van der Wal et al., 2001). GFP-PLC\(\delta_1\) was a kind gift of M. Katan (The Institute of Cancer Research, London, UK). Pi(4)P 5-kinase \(\alpha\) was kindly provided by N. Divecha (The Paterson Institute, Manchester, UK). The IP\(_3\) FRET sensor (Probe 0 269), the membrane-targeted FRB-CFP and mRFP-FKBP-IPP domain were kindly given by T. Balla (National Institutes of Health, Bethesda, USA). Yellow Cameleon Ca\(^{2+}\) sensor was provided by R. Tsien (Howard Hughes Medical Institute, La Jolla, USA).

Cell culture
Mouse N1E-115 neuroblastoma cells and N1E-115/TRPM7 cells were seeded on 24-mm glass-coverslips in 6-well plates (for microscopy) or in a 10-cm petridish (for biochemical assays) in DMEM supplemented with 10% FCS and antibiotics. Constructs were transfected using FuGene HD, at 1 mg DNA per well per construct or ~10 \(\mu\)g DNA per petridish. After 8-12 hours, the medium was refreshed.

Ca\(^{2+}\) imaging
For pseudo-ratiometrical Ca\(^{2+}\) recordings, cells on glass coverslips were incubated for 30 minutes with Fura Red-AM (2 \(\mu\)g/100 \(\mu\)l) and Oregon Green 488 BAPTA-AM (0.5 \(\mu\)g/100 \(\mu\)l), followed by further incubation in medium for at least 15 min (Langeslag et al., 2007; Rasmussen et al., 1986). Coverslips were mounted on a Nikon inverted microscope fitted with a Biorad MRC600 scanhead (Biorad, Herts, England). Recordings were made at 37°C in HEPES-buffered saline, composed of (in mM): NaCl (140), KCl (5), MgCl\(_2\) (1), CaCl\(_2\) (2), HEPES (10) and glucose (10), pH 7.2. Excitation of Oregon Green and Fura-Red was at 488 nm and single-cell fluorescence emission was detected at 522 ± 16 nm and at >585 nm, respectively. All Ca\(^{2+}\) recordings were normalized by setting basal levels at 1.0. For Ca\(^{2+}\) uncaging experiments, Iso-Ins(1,4,5)P\(_3\)/PM (caged) (2.0 \(\mu\)g per 100 \(\mu\)l) was co-loaded with Ca\(^{2+}\) dyes into cells. Uncaging of Iso-Ins(1,4,5)P\(_3\)/PM (caged) was achieved by a sub-second flash of UV light (355 ± 25 nM) from a mercury arc lamp. Excitation of eGFP was at 488 nm and emission was detected at 522 ± 16 nm.

Confocal imaging and deconvolution
Cells grown for 24 hours on glass coverslips were fixed for 10 minutes at room temperature in phosphate-buffered saline and 4% paraformaldehyde. Subsequently, cells were permeabilized with 0.1% triton X-100 in phosphate-buffered saline. Cells were incubated with rabbit anti-TRPM7 (1:500) followed by Alexa-488-conjugated anti-rabbit Ig (1:400) and Alexa-568-phalloidin (0.1 mg/100 ml). For actin staining in GFP-transfected cells, incubation with antibodies was omitted. The cells were mounted and placed on an inverted TCS-SP5 confocal microscope (Leica, Mannheim, Germany). For deconvolution, Z-stacks were recorded at z-intervals of 250-300 nm and deconvolved with Huygens deconvolution Software (Scientific Volume Imaging, Hilversum, the Netherlands).

Dynamic FRET essays
Cells grown on coverslips were transfected with FRET constructs (1 \(\mu\)g/coverslip) and experiments were performed as described previously (Ponsioen et al., 2004; van der Wal et al., 2001). In brief, coverslips were placed on an inverted Nikon microscope and excited at 425nm using an ND3 filter. CFP- and YFP emission were collected simultaneously through 470 ± 20 and 530 ± 25 nm bandpass filters. Data were acquired at 2 samples per second and FRET was expressed as ratio of CFP to YFP signals. This ratio was set to 1.0 at the onset of the experiments and changes are expressed as percent deviation from this initial value.
RNA interference and RT-PCR
Small-hairpin RNA constructs were generated by insertion (BglII/HindIII) of oligonucleotides into the pSuper vector and verified by sequencing analysis. Target sequences are listed in Table S1. Transfections were done using FuGene HD and a mix of pSuper vectors (listed in Table S1). Target sequences for PLCβ3 were a kind gift of W. Moolenaar (the Netherlands Cancer Institute, Amsterdam). For RT-PCR, RNA was collected from cells using RNA-Bee (AMS Biotechnology, Europe) according to manufacturers’ guidelines and cDNA was obtained using Superscript RNase H- Reverse Transcriptase (Gibco-Invitrogen). A subsequent PCR was performed using oligonucleotides listed in Table S2.

SDS-PAGE and immunoblotting
Cells were harvested in 1% NP40 lysis buffer, subsequently sample buffer was added and boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemiluminescence (Amersham Pharmacia).

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Supplemental Material

TRPM7-mediated Ca\(^{2+}\) influx in intact cells: Dual regulation by PIP\(_{2}\)

At first sight, the observations presented in Figures 1 and 2 would seem to conflict with the observed blockage of TRPM7 by PIP\(_{2}\), depletion in whole-cell patch clamp experiments (Gwanyanya et al., 2006; Langeslag et al., 2007; Macianskiene et al., 2008; Runnels et al., 2002). To further address this issue in intact cells, we employed the rapamycin-induced membrane recruitment of inositolpolyphosphate-5-phosphatase (IPP) (Suh et al., 2006; Varnai et al., 2006) to rapidly deplete the plasma membrane from PIP\(_{2}\) (see Fig. S1, next page). In these experiments, a membrane-bound, CFP-tagged FRB domain is coexpressed in cells with a mCherry-tagged IPP-FKBP chimera that is cytosolic and therefore inactive towards membrane PIP\(_{2}\). Addition of (membrane-permeable) rapamycin, which binds to FRB and FKBP simultaneously with high affinity, rapidly recruits the phosphatase to the plasma membrane to cause depletion of PIP\(_{2}\) by dephosphorylation at the 5'-position (Fig. S1A, middle panel photomicrographs). Indeed, administration of rapamycin during the BK-induced sustained Ca\(^{2+}\) phase caused influx to rapidly decline (Fig. S1A, top panel, gray trace). In control N1E-115/TRPM7 cells expressing either one of the constructs alone, rapamycin had no effect (data not shown). TRPM7 closure was also observed when PIP\(_{2}\) was depleted by interfering with its resynthesis using high levels (1 µM) of Wortmannin (Suh and Hille, 2002) (Fig. S1B). Thus, in intact cells too, Ca\(^{2+}\) assays show that near-complete depletion of PIP\(_{2}\) inhibits the channel, in agreement with the earlier mentioned whole-cell patch clamp experiments. Collectively, these data show that TRPM7 is dually regulated by membrane PIP\(_{2}\): whereas a drop in PIP\(_{2}\) levels is essential for TRPM7 activation, the near-complete depletion of this lipid blocks channel functioning altogether. Put in other words, a limited range of PIP\(_{2}\) levels supports TRPM7 conductance. Note that biphasic PIP\(_{2}\) dependency has also been found for for example TRPV1 (Lukacs et al., 2007; Lukacs et al., 2013) and TRPM8 (Rohacs et al., 2005; Yudin et al., 2011). It is interesting to note that the block of TRPM7 following near-complete PIP\(_{2}\) breakdown may constitute a negative feedback mechanism: when PIP\(_{2}\) levels drop below a critical threshold, TRPM7 closes and further PIP\(_{2}\) breakdown would be prevented. See next page for Fig. S1.

Supplemental Table 1 | RNA interference target sequences.

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<th>Sequence #1</th>
<th>Sequence #2</th>
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<td>CTGATCCTCAAGACGTTAA</td>
</tr>
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<td>CCGGCTCTTCGTCTTCC</td>
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<tr>
<td>PLC(\delta)_1</td>
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<td>PLC(\delta)_2</td>
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Supplemental Table 2 | RT-PCR oligonucleotides.

<table>
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<th>Rv</th>
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<tr>
<td>PLC(\eta)_2</td>
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<td>PLC(\gamma)_2</td>
<td>GCTTGCGAAAGGCTGATAA</td>
<td>CCGGCTCTTCGTCTTCC</td>
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See next page for Fig. S1.
Supplemental Figure 1 | Dependence of TRPM7-mediated Ca\(^{2+}\) influx on membrane PIP\(_2\). (A) TRPM7-mediated sustained Ca\(^{2+}\) influx is blocked upon depletion of PIP\(_2\). Addition of rapamycin recruits inositolpolyphosphate-5-phosphatase (IPP) to the membrane and disrupts the sustained Ca\(^{2+}\) phase (gray trace, ‘s1-3’), whereas the response in a neighboring untransfected cell is unaffected (black trace). Middle panel: GFP-PH labeling of a cell before (‘b’, left) and 2 minutes after rapamycin addition (‘s2’, right) shows complete loss of membrane PIP\(_2\). Typical example of N = 34. (B) BK-induced Ca\(^{2+}\) responses in cells pretreated with Wortmannin. Treatment with 1 µM to inhibit PIP-4-kinase causes depletion of PIP\(_2\) within ~ 5 min (middle panel; ‘s3’, right versus ‘b’, left) and completely blocks the sustained Ca\(^{2+}\) phase (gray trace, ‘s1-3’). As a control, treatment with 50 nM Wortmannin, which blocks PI 3-kinase but leaves PIP-4-kinase activity unaltered, was without effect (black trace) (N = 6 cells). Quantifications are depicted in the bar graphs in the lower panels of both A and B. * and #, p < 0.001 and p < 0.003 compared to respective basal values. ‘b’, basal; ‘s1-3’, time points during sustained phase.