

A painful TR(i)P to lysosomes

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The ion channel TRPA1 detects noxious stimuli at the plasma membrane of neurons and elicits pain and inflammation. In this issue, Shang et al. (2016. *J. Cell Biol.* <http://dx.doi.org/10.1083/jcb.201603081>) report that TRPA1 also localizes to lysosomal membranes of neurons, releasing intracellular Ca^{2+} to trigger vesicle exocytosis and neuropeptide release.

Transient receptor potential (TRP) channels are a superfamily of Ca^{2+} -permeable cation nonselective channels with diverse functions. A subset of TRPs is expressed at the plasma membrane of somatosensory neurons serving as molecular sensors for diverse environmental stimuli such as temperature, mechanical force, and plant-derived chemicals (Julius, 2013). The most well studied are TRPV1, the heat and capsaicin receptor, and TRPA1, the receptor for wasabi (i.e., mustard oil or allyl isothiocyanate [AITC]). TRPA1 is activated by structurally diverse noxious compounds, including AITC, and other electrophilic compounds in pungent spices, as well as environmental irritants such as tear gas (Bautista et al., 2006; Macpherson et al., 2007; Julius, 2013). TRPA1 has a twofold contribution to neurogenic inflammation. First, activation of TRPA1 increases intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) to facilitate dense-core vesicle (DCV) exocytosis and release of inflammation-inducing neuropeptides, such as calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY), which in turn enhance the excitability of primary nociceptive neurons that cause pain (Bautista et al., 2006). Second, upon activation of G protein-coupled receptors by itch-inducing substances and inflammatory mediators such as bradykinin, TRPA1 channels are activated by signal transduction pathways downstream of phospholipase C (Bautista et al., 2006; Wilson et al., 2011). Whereas TRPA1 knockout (KO) mice exhibit reduced inflammatory responses toward noxious stimulations (Bautista et al., 2006), gain-of-function mutations in human TRPA1 cause familial episodic pain syndrome (Kremeyer et al., 2010). Hence, TRPA1 represents a promising drug target for pain, itch, and other chronic inflammation-related disorders such as asthma (Bautista et al., 2006; Wilson et al., 2011; Julius, 2013).

In addition to their plasma membrane localization, many TRPs are also found in intracellular membranes (Dong et al., 2010). For example, TRPV1, TRPM8, and TRPP2 are localized on ER membranes, whereas TRPC3, TRPC5, TRPV5-6, TRPM2, TRPM8, TRPA1, and TRPML1-3 are present in secretory vesicles; early and recycling endosomes; and lysosomes (Dong et al., 2010; Fig. 1). Unlike Mucolipin TRPs (TRPMLs), which predominantly localize at endosomes and lysosomes (Xu

and Ren, 2015), the other TRPs are thought to be plasma membrane channels that temporarily associate with intracellular membranes as “cargos” during their biosynthesis and secretion (Dong et al., 2010). To avoid misregulation of membrane traffic and organellar ion homeostasis, these cargos are kept inactive until they reach their destinations (Dong et al., 2010); for example, cofactors required for plasma membrane channel function such as phosphatidylinositol 4,5-bisphosphate ($\text{PI}(4,5)\text{P}_2$) are less abundant or absent from intracellular membranes (Xu and Ren, 2015). TRPs in acidic vesicles might also be inhibited by low luminal pH, and ER-localized TRPs might be inactive because of the lack of glycosylation that occurs in the Golgi membrane after ER exit (Dong et al., 2010).

In this issue, Shang et al. made an unexpected observation while performing a negative control experiment in their study of TRPA1-mediated Ca^{2+} responses: Complete removal of external Ca^{2+} failed to abolish AITC-induced $[\text{Ca}^{2+}]_i$ increase and neuropeptide release in DRG neurons. Blocking TRPA1 channels with ruthenium red, a membrane-impermeable blocker, was also ineffective. However, the AITC-induced responses were entirely TRPA1 specific, as they were abolished in DRG neurons from TRPA1 KO mice or by membrane-permeable synthetic inhibitors of TRPA1 (Paulsen et al., 2015). Given that ruthenium red also blocks TRPA1-mediated Na^+ influx, the AITC-induced Ca^{2+} increases in zero external Ca^{2+} were unlikely to be caused by $[\text{Ca}^{2+}]_i$ release triggered by membrane depolarization. These results were surprising because they suggest that the responses to AITC were mediated directly by intracellular TRPA1 channels.

To investigate the functional sites of intracellular TRPA1 channels, Shang et al. (2016) first used pharmacological inhibitors to rule out the involvement of the ER and mitochondria, common Ca^{2+} stores or sinks in the cell. The V-ATPase inhibitor Baf-A1 completely abolished the responses, which suggested that the source of AITC-induced Ca^{2+} release was most likely from acidic stores/vesicles. This observation is consistent with previous studies showing that acidified vesicles are also $[\text{Ca}^{2+}]_i$ stores with the luminal Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{lumen}}$) estimated to be ~ 0.5 mM for lysosomes (Christensen et al., 2002). Furthermore, glycyl-L-phenylalanine 2-naphthylamide, a cathepsin-C substrate that permeabilizes lysosomal membranes (Garrity et al., 2016), abolished TRPA1-mediated Ca^{2+} release. Hence, the $[\text{Ca}^{2+}]_i$ stores on which TRPA1 agonists act are lysosomes or lysosome-related compartments. Indeed, immunofluorescence in DRG neurons showed that TRPA1 colocalized with lysosome-associated membrane protein 1 (Lamp1), a marker

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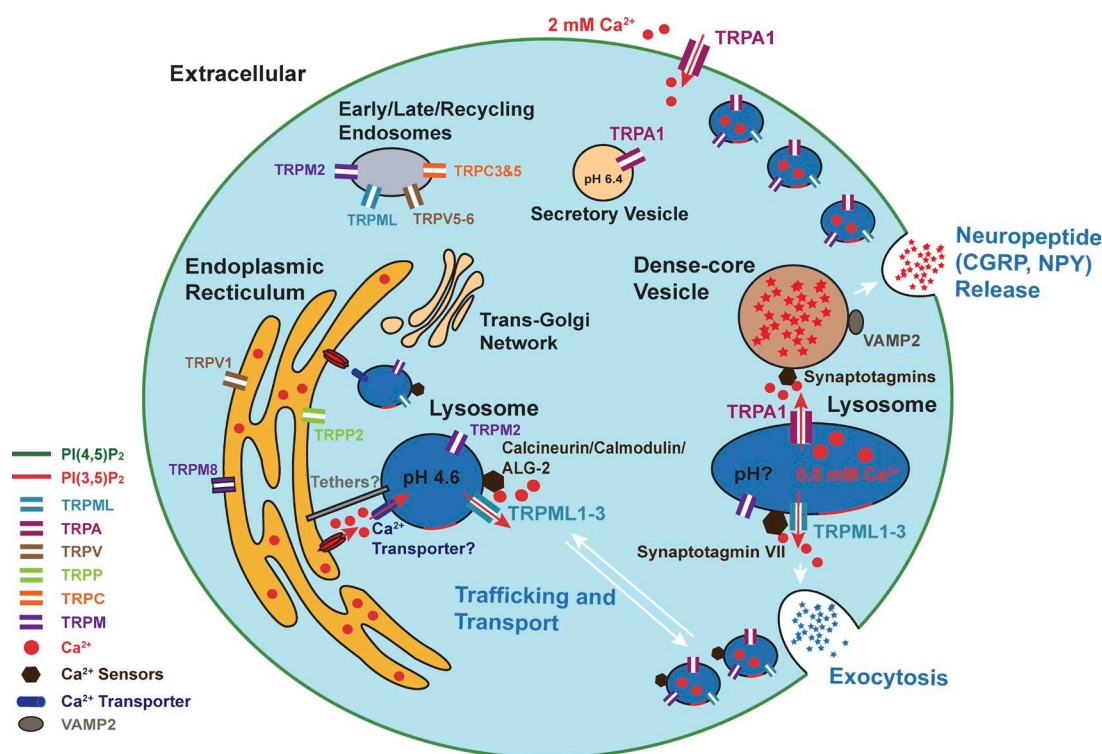


Figure 1. TRPA1 is a lysosomal Ca^{2+} release channel in DRG neurons. Several TRP channels are localized at both plasma membranes and intracellular membranes, such as ER, secretory vesicles, endosomes, and lysosomes. The compositions of lipids (PI(3,5)P₂ for lysosomes and PI(4,5)P₂ for the plasma membrane) and luminal ions are indicated for each compartment. In the DRG neurons, TRPA1 has a dual location: plasma membrane (PI(4,5)P₂ enriched) and lysosomes. Upon activation by TRPA1 agonists such as AITC, plasma membrane TRPA1 mediates Ca^{2+} influx and lysosomal TRPA1 mediates Ca^{2+} release from lysosomes ($[\text{Ca}^{2+}]_{\text{lumen}}$ is ~ 0.5 mM) at the periphery of the cell near plasma membrane. Ca^{2+} release through TRPA1 activation is sufficient to activate Ca^{2+} sensors (e.g., synaptotagmins) in the VAMP2-localized DCVs, triggering DCV exocytosis and neuropeptide (CGRP and NPY) release. TRPMLs are present on all lysosomes (pH 4.6; $[\text{Ca}^{2+}]_{\text{lumen}}$ is ~ 0.5 mM Ca^{2+} ; PI(3,5)P₂), mediating Ca^{2+} -dependent lysosomal exocytosis, membrane trafficking, and transport through Ca^{2+} sensors (calcineurin/ALG-2/CaM/synaptotagmins). In the cytoplasm, the ER may provide Ca^{2+} to lysosomes through ER–lysosome membrane contact sites.

for late endosomes and lysosomes. The colocalization was most prominent at the periphery of the cell near the plasma membrane, but less so on the vesicles in the bulk of the cytosol. Immunogold labeling revealed that TRPA1 was actually localized on the limited/perimeter membranes of Lamp1 compartments near the plasma membrane. Lysosomes are heterogeneous in size and their distribution in the cell is highly regulated (Xu and Ren, 2015; Li et al., 2016). A recent study suggests that peripherally localized lysosomes are less acidic (Johnson et al., 2016). Shang et al. (2016) used line-scan confocal imaging to show that Ca^{2+} release was from individual Lamp1-positive areas near the plasma membrane. Whole-endolysosome patch-clamp recording techniques have been developed to study acidic organellar channels with the aid of agents such as vacuolin compounds to enlarge lysosomes (Xu and Ren, 2015). Vacuolin-1 was ineffective in enlarging the TRPA1 lysosomes, but future studies of their electrophysiology could use other methods (e.g., PI(3,5)P₂ deficiency causes lysosome enlargement in DRG neurons [Zhang et al., 2007]). Despite the lack of lysosomal electrophysiology, the collective evidence nevertheless strongly supports the functional expression of TRPA1 in the peripheral lysosomes of DRG neurons (Fig. 1).

To dissect the relative contribution of extracellular versus lysosomal sources of Ca^{2+} , Shang et al. (2016) developed a “ ΔF d analysis” method and estimated that lysosomal Ca^{2+} release accounted for $\sim 40\%$ of the total $[\text{Ca}^{2+}]_{\text{i}}$ increase in the initial phase, which may be an overestimate given that the total lysosomal

membrane is only $\sim 1\%$ of the plasma membrane. Another consideration is that lysosomal Ca^{2+} release is dependent on the store refilling and TRPA1 lysosomal stores appeared to be quickly depleted by AITC. In contrast, Ca^{2+} influx is not limited by the supply of extracellular Ca^{2+} (2.0 mM). Interestingly, in terms of physiological output (i.e., Ca^{2+} -dependent vesicle exocytosis and neuropeptide secretion), lysosomal TRPA1 has a significant contribution, suggesting that lysosomal Ca^{2+} release rather than Ca^{2+} influx might be preferentially coupled with DCVs (Fig. 1). The relative physiological contribution of lysosomal versus plasma membrane TRPA1 could perhaps be investigated in the future by targeting Ca^{2+} sensors to DCV-specific proteins (Fig. 1). To measure the physiological consequences of TRPA1 activation in DRG neurons, Shang et al. (2016) used three independent approaches. First, vesicle exocytosis was monitored using real-time capacitance recording. Second, total internal reflection fluorescence microscopy was used to measure the fluorescence of NPY-pHluorin, which became dequenched upon exocytosis. Lastly, CGRP release was directly measured using ELISA. In all assays, substantial AITC-induced release was maintained in the absence of external Ca^{2+} , but was abolished by TRPA1 KO. Shang et al. (2016) studied the cell bodies of newly dissociated DRG neurons, but it is worth noting that DCVs may be localized in the presynaptic or extrasynaptic regions where TRPA1-localized lysosomes are clustered and surface expression of TRPA1 is low.

Lysosomal Ca^{2+} release can also trigger lysosomal exocytosis and regulate lysosomal membrane trafficking, including

membrane fusion, fission, and transport (Xu and Ren, 2015; Li et al., 2016). Multiple Ca^{2+} sensors are implicated in these processes, including synaptotagmins (Syt-VII), calcineurins, calmodulins (CaM), and ALG-2 (Xu and Ren, 2015; Fig. 1). It remains to be determined whether activation of lysosomal TRPA1 induces lysosomal exocytosis. In mast cells, TRPA1, localized in the secretory vesicles, reportedly undergoes Ca^{2+} -regulated exocytosis (Prasad et al., 2008). Lysosomal exocytosis may release protons and luminal enzymes, such as cathepsin proteases, which promote inflammation. TRPML1-mediated lysosomal Ca^{2+} release is known to regulate lysosomal exocytosis, trafficking, and transport (Xu and Ren, 2015). It will be interesting to investigate whether lysosomal TRPA1 has a similar role in general membrane trafficking. However TRPA1 lysosomes are not enlarged by vacuolin-1, suggesting that membrane trafficking of TRPA1 lysosomes is not or differentially regulated by general trafficking cues (Fig. 1). No autophagic defects or lysosomal dysfunction have been reported for TRPA1 KO mice. A provocative speculation is that peripherally localized TRPA1 lysosomes are selectively connected with the neurophysiological functions but not lysosomal and cell biological functions in DRG neurons.

The functional presence of TRPA1 in the lysosomes of DRG neurons, but not in HEK cells or DRG-derived neuronal cell lines, suggests that TRPA1 is targeted to lysosomes via a mechanism that is specific to primary neurons. A common misconception among channel biologists is that lysosomes are the “destiny” of most membrane proteins. Hence, it is no surprise that some ion channels are detected in the lysosomes. However, all proteins destined for degradation localize in the intraluminal vesicles inside the lysosome (i.e., multivesicular bodies), but not on the limited/perimeter membrane where all resident lysosomal channels/transporters are localized. Unlike resident membrane proteins with a slow turnover time (days), destined-to-be-degraded membrane proteins are ubiquitinated and have very short half-lives (hours) in the lysosome (Li et al., 2015). Although destined-to-be-degraded proteins are in the “passenger’s seat” of endocytosis, lysosome-localized membrane proteins, including TRPML1 in all cell types and lysosomal TRPA1 in DRG neurons, are in the “driver’s seat.” Hence, active lysosomal targeting mechanisms must exist for TRPA1 in DRG neurons. TRPMLs and two-pore channels are targeted to lysosomes via di-leucine motifs (Xu and Ren, 2015). The fact that TRPA1’s localization is preferentially restricted to peripheral lysosomes in the DRG neurons suggests the existence of additional mechanisms other than the general di-leucine motif. It would be interesting to test if ubiquitylation of lysosomal TRPA1 is misregulated in primary DRG neurons (Li et al., 2015), thus increasing the dwelling time of TRPA1 in the limited membrane. If DRG-specific lysosomal targeting mechanisms exist, it would be interesting to separate the effects of plasma membrane versus lysosome targeting on neuropeptide release. More importantly, the role of lysosome targeting of TRPA1 should be investigated in neurogenic inflammation mediated by endogenous inflammatory mediators. Finally, lysosome–ER contact was recently proposed to regulate lysosomal Ca^{2+} refilling (Garrity et al., 2016). ER tubules are less abundant at the periphery of the cell, which may explain the lack of Ca^{2+} refilling in TRPA1 lysosomes. However, the lack of refilling might also be a result of persistent activation of lysosomal TRPA1 by electrophile-mediated covalent modification. Hence, in future studies, lysosomal Ca^{2+} release, channel

desensitization, and store refilling should also be studied and compared using nonelectrophile agonists such as carvacrol.

The study by Shang et al. (2016) raises the question of whether and how TRPA1 channels might be differentially regulated in the plasma membrane versus lysosomes. AITC is a potent agonist and may override the endogenous inhibitory mechanisms. Plasma membrane TRPA1 is modulated by a variety of cellular cues, for instance, G protein and lipid and Ca^{2+} signaling. Strikingly, whereas plasma membrane TRPA1 is potentially activated by Ca^{2+} (Zurborg et al., 2007), lysosomal TRPA1 is not. There are several possible explanations for this observation. First, the lipid composition of lysosomes is unique. For example, they lack $\text{PI}(4,5)\text{P}_2$, which may be required for TRPA1 function, and lower the sensitivity of lysosomal TRPA1 to cellular cues. TRPML1 is activated by $\text{PI}(3,5)\text{P}_2$, a lysosome-specific phosphoinositide (Zhang et al., 2012). It remains to be determined whether $\text{PI}(3,5)\text{P}_2$ or other lysosomal lipids may serve as a positive regulator of lysosomal TRPA1. Second, a salient feature of lysosomes is their low pH, which may inhibit mouse and rat TRPA1 (de la Roche et al., 2013). Interestingly, human TRPA1 is activated by low pH (de la Roche et al., 2013), raising the possibility that lysosomal TRPA1 might play a bigger role in neurogenic inflammation in humans. However, the acidification of peripheral lysosomes in DRG neurons (Fig. 1) is an assumption at present, which requires experimental verification. Third, the lysosomal membrane potential ($\Delta\psi$ approximately -30 mV, luminal-side positive) is less negative than the resting membrane potential of the cell ($\Delta\psi$ approximately -70 mV), providing less driving force for Ca^{2+} fluxes (Dong et al., 2010; Xu and Ren, 2015). In addition, the voltage dependence of TRPA1 activation may also play a role in lysosomal Ca^{2+} release (Paulsen et al., 2015). Because drugs like Baf-A1 and glycyl-L-phenylalanine 2-naphthylamide are known to affect lysosomal $\Delta\psi$, caution is necessary when interpreting the observed effects of these compounds on lysosomal Ca^{2+} release. In contrast, other lysosome-relevant cellular factors may favor TRPA1 activation. For example, the level of reactive oxygen species, known agonists of TRPA1 (Miyake et al., 2016), is expected to be high in the lysosome lumen.

In summary, the study by Shang et al. (2016) used mouse KOs and various pharmacological reagents to convincingly show that TRPA1 mediates ER-independent Ca^{2+} release from the lysosome-like vesicles, which are predominantly distributed in the periphery of DRG neurons, and raises many important questions. For example, does cell-specific targeting to intracellular membranes confer cell type-specific functions? Are such targeting mechanisms regulated by cellular cues? What are the physiological functions of other intracellularly localized TRPs including TRPV1 and TRPM8? Finally, are intracellularly localized TRPs regulated by organellar $\Delta\psi$, and organelle-specific luminal and cytosolic factors? Answering these questions may open a new era for organellar channel research.

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