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Review

Mechanisms of Activation of LRRC8 Volume Regulated Anion Channels

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Abstract
Volume regulated anion channels (VRACs) are ubiquitously expressed in all vertebrate cells. Despite many years of research, the fundamental mechanisms underlying VRAC activation are not understood. The recent molecular identification of the LRRC8 genes underlying VRAC revealed that VRACs are formed by a hexameric assembly of members of the LRRC8 gene family. Knowing the genes underlying VRACs allowed the discovery of novel VRAC functions into cell volume regulation, and first structure function studies revealed important insight in channel activation mechanisms. The determination of cryo-EM structures of homomeric LRRC8A and LRRC8D complexes provide a framework for a rational approach to investigate biophysical mechanisms. We discuss several recent advances within the structural framework, and we critically review the literature on the main mechanisms proposed to be involved in VRAC activation, including low intracellular ionic strength, membrane unfolding, oxidation, phosphorylation and G-protein coupling.

The structure of LRRC8 VRAC channels

The composition of the intracellular milieu is vastly different from that of the immediately adjacent extracellular environment in cells from all organisms. Highly specialized transmembrane proteins determine the tightly controlled transport of substrates across the plasma membrane. In this review we are considering transport mediated by so-called volume regulated anion channels (VRACs). A most intriguing feature of VRACs is their involvement in disparate processes including volume regulation, cell to cell signaling, metabolic sensing, apoptosis, membrane potential regulation, and chemotherapeutics uptake.

Anion currents activated by extracellular hypotonicity have been first described by Lewis and Cahalan in T lymphocytes [1] and by Hazama and Okada in intestinal epithelial cells [2]. Since then, similar "volume regulated anion channels" (VRACs) have been described and

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extensively characterized in practically all vertebrate cell types [3-6]. Several names have been assigned to such hypotonicity activated anion currents [3, 6]. Here, for simplicity, we will stick to the "VRAC" nomenclature, and refer to the reviews by Jentsch and Strange et al. regarding other nomenclatures [3, 6].

In 2014 two independent groups identified the "Leucine Rich Repeat Containing 8 A" (LRRC8A) gene as encoding an essential VRAC subunit [7, 8]. Voss et al. additionally showed that VRACs are heteromeric proteins that contain the LRRC8A subunit in an obligatory manner, and additionally one or more subunits encoded by the homologous genes LRRC8B-E [7]. Single-molecule photo bleaching studies and biochemical assays suggested that the stoichiometry is not fixed and that VRAC channels may be composed of more than two different subunits [9, 10]. LRRC8 proteins are about 800 amino acids long. The first ~400 amino acids are organized in four transmembrane domains and show homology to pannexin channel subunits [11], while the last ~400 amino acids are unique to LRRC8 proteins and contain 17 cytosolic leucine rich repeats (LRRs) [11]. Four different high-resolution cryo-EM structures of homomeric LRRC8A assemblies and lower resolution structures of LRRC8A-LRRC8C heteromers [12-15] fully confirmed the predictions of Abascal & Zardoya [11]. Three of these structures were obtained from detergent solubilized proteins [12-14]. The structure obtained by Deneka et al. [12] is illustrated in Fig. 1A. The protein can be divided in several domains including an extracellular domain (ED), which is not fully resolved, and in which several beta strands are stabilized by intra-subunit disulfide bonds. The ED forms the most narrow part of the pore. The transmembrane domain (TMD) is formed by four helices (TM1-TM4) from each subunit with TM1 and TM2 contributing to pore lining with mostly hydrophobic residues. The ED and TMD exhibit six-fold symmetry as illustrated in Fig. 1B and the protein fold is similar not only to that of pannexins but also to that of connexins, even though there is no sequence homology with connexins [11, 12]. The cytosolic subdomain (CSD) is located below the membrane and is formed by the intracellular loop connecting TMD2 and TMD3 and the stretch connecting TMD4 with the leucine rich repeat domain (LRRD). The CSD exhibits a complex fold and the six fold symmetry of the ED and TMD is broken at the level of the CSD resulting in a three-fold symmetry at the level of the LRRD (according to three out of four published structures - see below), which form a trimer of dimers (Fig. 1C). Several, possibly important parts of the protein are not resolved, including the first 14 residues at the N-terminus, a 23 amino acid stretch of the ED and a relatively long stretch in the intracellular loop connecting TMD2 and TMD3 in the CSD [12]. All three

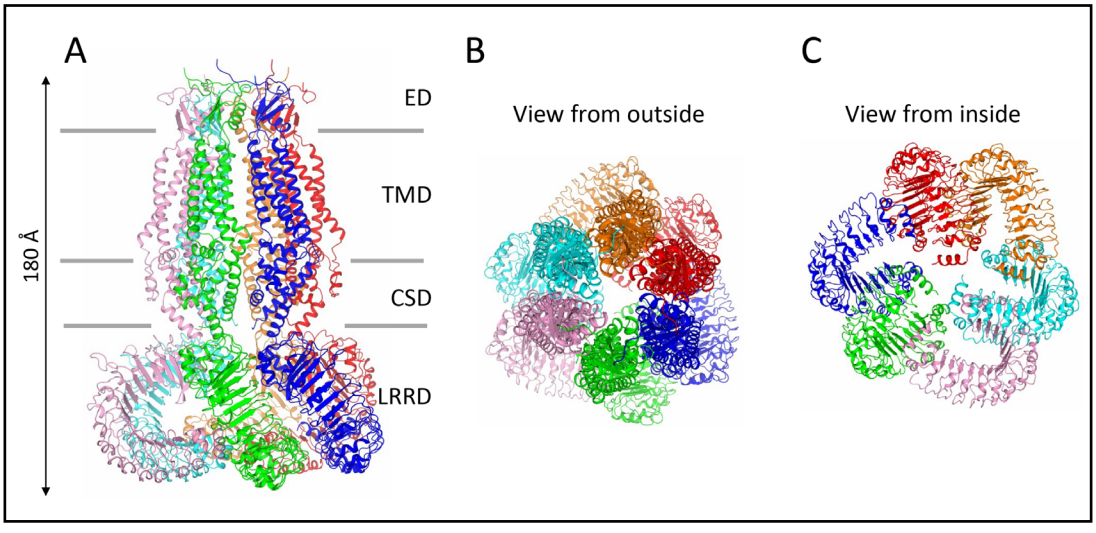

Fig. 1. Structure of homomeric LRRC8A. The structure of homomeric mouse LRRC8A (pdb 6g9l; [12]) is shown from different views. Each subunit is shown in a different color (ED, extracellular domain; TMD transmembrane domain; CSD, cytoplasmic subdomain; LRRD, leucine rich repeat domain).

structures obtained using detergent solubilized protein are similar [12-14]. The structures reported by Kern et al. exhibit some differences to these and were obtained using protein incorporated into lipid nanodiscs [15]. Classification of the cryo-EM images predicted two different structures, a "constricted" structure and a "relaxed" structure, which might be related to gating movements [15]. Surprisingly, the "nanodisc structures" are considerably different from the "detergent structures" in two key aspects. First, in the nanodisc structures the TMD is more loosely packed, with several lipid molecules intercalating into the membrane pore wall. Second, the nanodisc structures are overall six-fold symmetric, *i.e.* do not show a trimer of dimers configuration of the LRRD [15]. Which of the two classes of structures is more relevant to native heteromeric LRRC8 structures remains to be determined.

It needs to be underlined that all four high resolution structures are obtained from non-physiological homomeric LRRC8A complexes. Even though homomeric LRRC8A channels exhibit residual function [12, 13, 15], the functional properties appear to be quite different from those of physiological VRACs [16]. For example currents obtained from LRRC8 KO cells transfected with only LRRC8A are poorly volume sensitive and also much less sensitive to low intracellular ionic strength than regular VRAC channels [16, 17]. Interestingly, Yamada & Strange found that homomeric functional volume-sensitive channels could be formed by LRRC8C, D or E subunits in which the intracellular loop connecting TMD2 and TMD3 was replaced with that from LRRC8A [16]. The necessary region to achieve functional homomeric channels could be narrowed down to the stretch from residues D182 to D206. Such a homomeric construct could be useful to obtain a structure of a functional homo hexameric channel.

More recently the structure of a homomeric LRRC8D assembly was resolved using cryo-EM of protein solubilized in detergent [18]. Even though the overall architecture is similar to that of the homomeric LRRC8A structures, the homo-hexameric LRRC8D structure exhibits 2-fold dimer of trimers symmetry, both in the membrane as well as the intracellular domains [18]. The outer pore entry is wider compared to the structures of homomeric LRRC8A, consistent with the higher permeability of LRRC8D containing heteromeric LRRC8A/LRRC8D complexes for organic osmolytes [9, 19, 20]. Yet, similar to the homomeric LRRC8A structures, the relevance of the homomeric LRRC8D structure remains to be determined.

Emergent physiological roles of LRRC8 VRAC channels

The fact that VRACs are activated by hypotonicity indicates that the channels are important for cellular volume regulation, and indeed pharmacological inhibition impairs the ability of cells to achieve a regulatory volume decrease (RVD) [3, 4, 21]. This result was fully confirmed in LRRC8A knock-out cells [7]. In addition to chloride, VRACs are permeable to small organic, mostly anionic or uncharged osmolytes like taurine [4, 6, 9, 10, 22]. This permeability likely plays an important role in volume regulatory processes like RVD or apoptotic volume decrease (AVD) [23]. In addition, release of excitatory amino acids or ATP through VRAC channels has been proposed to be important for signaling and toxicity in glia and other cell types [24-26].

Mice in which *Lrrc8a* is knocked out are characterized by increased mortality before and after birth and have several severe other defects including growth retardation and defective lymphocyte function [27]. However, the severity of the phenotype allows little conclusions because indirect effects cannot be distinguished from specific ones. Since the discovery that LRRC8 proteins constitute VRAC, more specific knockout strategies have revealed novel functions of LRRC8 proteins. Several of these have been excellently reviewed by Chen et al. [28]. Among others, these include resistance to cancer drugs [20, 29-31], insulin secretion [32, 33], spermiogenesis [34, 35], astrocyte mediated signaling [36-38], and ATP release [39]. In astrocytes, an additional role has been proposed regarding the interaction of LRRC8 with the cell adhesion molecule GlialCAM and the membrane protein of unknown function MLC1, mutations of which lead to megalencephalic leukoencephalopathy with subcortical cysts (MLC) [40].

More recently discovered roles include myogenesis [41, 42], glucose metabolism [42], reduction of intracellular chloride concentration [43], release and uptake of cyclic di-nucleotides, which are important in the STING defense pathway [44, 45], and the permeability to glutathione proposed to modulate epithelial-to-mesenchymal transition [46]. Interestingly, the involvement of VRAC in migration and proliferation [3, 4] could not be confirmed in several cell lines [47]. This important aspect requires further investigation. Further physiological roles of VRAC, possibly related to specific LRRC8 subunits, are likely to emerge in the future.

Several functional roles of LRRC8 proteins appear to be related to VRAC's ability to decrease cell volume by allowing chloride and osmolyte efflux. For example, in spermiogenesis, cells have to reduce their volume and LRRC8A defects lead to swollen plasma and deformations of late spermatids [34]. In contrast, other functions appear to be unrelated to changes in cell volume but closely linked to the ability of VRACs to translocate specific large anionic molecules, like cyclic di-nucleotides [44, 45] or excitatory amino acids [36, 37].

In any case, it is clear that cells have to control tightly the activity of VRAC, because excessive activity would lead to the loss of cellular small organic molecules and would induce cell shrinkage. Indeed, transfection of constitutively active VRAC constructs lead to shrunken cells that could not anymore be patch-clamped [16]. In addition, an unexpected role of VRAC for lysosomal ion homeostasis was recently proposed [48], and this finding significantly extends the possible roles of VRAC for cellular physiology.

Surprisingly, despite these important physiological and pathophysiological insights, and the necessity of cells to tightly control VRAC activity, the fundamental question of which are the molecular and cellular mechanisms that underlie the activation of VRAC are incompletely understood. One of the few certain aspects regarding these mechanisms is that VRAC activation does not involve exocytotic insertion of "novel" VRAC channels into the plasma membrane [49]. In the following sections we first review which structural parts of VRAC proteins have been implicated in channel activation, and finally we provide an overview on the proposed mechanisms of VRAC activation and modulation in various cell types.

Structural parts of LRRC8 proteins involved in channel activation

Classically, ion channel functionality is described in terms of "closed" and "open" states, *i.e.* the channel can be in a non-conducting, closed state or in a partially or fully open state [50]. This paradigm has been confirmed for VRAC channels both in native systems and in heterologous expression systems by single channel recordings [9, 51-55]. The transition between closed and open states, called channel activation or channel gating, necessarily implies conformational changes of the channel protein complex. The question of how VRAC activation occurs can be further divided into three overlapping sub-questions:

1) Which protein domains are critically important for channel activation?
2) Which are the relevant cellular stimuli for VRAC activation?
3) How are these cellular stimuli conveyed to the relevant protein segments?

We first summarize the current knowledge on protein domains implicated in channel gating.

An extracellular region involved in channel inactivation

A distinctive feature of VRACs composed of different subunits is the degree and kinetics of current inactivation at positive voltage, as illustrated in Fig. 2. LRRC8A/LRRC8E complexes exhibit profound and rapid inactivation at positive voltages [7, 9] (Fig. 2A), similar to what is seen for example in endogenous VRAC in glioblastoma cells [56] (Fig. 2D), and also in several others. Conversely, LRRC8A/LRRC8D channels exhibit similar but slower and less

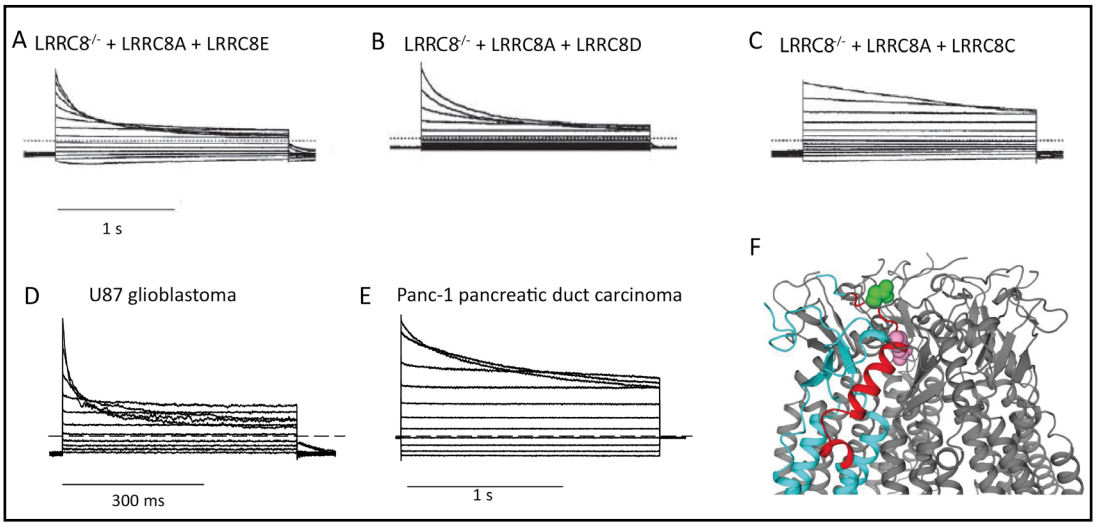

Fig. 2. Subunit composition determines channel inactivation kinetics. Panels A-C illustrate different inactivation kinetics of currents measured in LRRC8 knockout cells transfected with the indicated subunits (modified from [7]; applied voltages range from -120 to 120 mV). In native cells, inactivation kinetics of VRAC is variable between cell types, but consistent for a given cells type. Panels D and E show example recordings from U87 glioblastoma cell with extremely fast inactivation, from a Panc-1 (pancreatic duct cancer cell line) cell with very slow inactivation (applied voltages range from -120 to 120 mV). Panel F highlights the structural loops identified by Ullrich et al. [19] to be determinants of inactivation kinetics. One of the 6 subunits is shown in light blue, expect the C-terminal half of the first extracellular in red. The residue corresponding to D102 of LRRC8C that is a major determinant of inactivation [19] is shown in space fill in green. It is the most "extracellularly" exposed residue. R103, which defines the narrowest part of the pore is shown in space fill in pink (pdb: 6djb, [13]).

pronounced inactivation [7, 9] (Fig. 2B), which further decreases in LRRC8A/LRRC8C heteromers [7, 9] (Fig. 2C), similar to VRAC channels in lymphocytes [57, 58] or Panc-1, a cell line derived from pancreatic duct carcinoma (Fig. 2E).

The physiological relevance of the inactivation is unclear, however the comprehension of this phenomenon is important to elucidate the general gating mechanisms of VRAC. Exploiting the kinetic differences between LRRC8A/8E and LRRC8A/8C heteromers and employing a chimeric strategy, Ullrich et al. identified the C-terminal half of the first extracellular loop as a major determinant of VRAC inactivation [19]. In Fig. 2F the corresponding region is highlighted in red in one LRRC8A subunit of the homomeric structure determined by Kefauver et al. [13]. The residue R103, which defines the narrowest part of the pore, is highlighted in pink and the residue corresponding to D102 of LRRC8C, the most "extracellularly" exposed residue within the resolved part of the homomeric LRRC8A structure and a major determinant of inactivation [19], is highlighted in green (Fig. 2F). Thus, the functional results of Ullrich et al. [19] and the structural information suggest that inactivation involves a conformational change of the external pore constriction. We speculate that inactivation reflects a closure of the outer pore entrance. Whether positive voltages induce inactivation directly by acting on charged VRAC protein segments, or indirectly, for example by interaction of permeating substrates with the protein remains to be investigated.

While inactivation might be relevant to determine the degree of VRAC channel activity in specific physiological settings, it is unlikely that the inactivation process is mechanistically related to the general mechanism of VRAC activation by hypotonicity. However, as we still don't know the basic VRAC activation mechanism, a possible involvement of the inactivation process should be kept in mind.

Activation involves a separation of the C-terminal LRRDs

Compared to connexins and pannexins, a unique structural feature of LRRC8 subunits is their extended C-terminal cytosolic LRRD. Thus, since channels formed by LRRC8 subunits are volume sensitive, while connexins and pannexins are not, it is likely that the LRRDs are involved in VRAC activation. Two lines of evidence suggest indeed that channel activation is accompanied by a widening, *i.e.* increased spatial separation of the LRRDs. First, attaching fluorescent proteins like GFP or mCherry to the C-terminus of LRRC8 subunits resulted in channels that showed a constitutive activity in the absence of hypotonic stimulation [9, 59]. Addition of such ~27 kD proteins to each of the six subunits is expected to induce significant sterically induced displacement of the C-termini. Fig. 3 highlights the relative size of a GFP molecule with respect to the LRRDs. Importantly, these tagged channels could be further stimulated by hypotonicity demonstrating that tagging did not fully open the channels and that the basic mechanism of channel activation remained intact. Interestingly, attaching a 3xHA epitope to the C-termini led to a much smaller activation [9] (see Fig. 3 for a comparison in size between a 3xHA epitope and GFP), thus suggesting that the size of the tag, and the degree of LRRD separation determine the degree of channel activation.

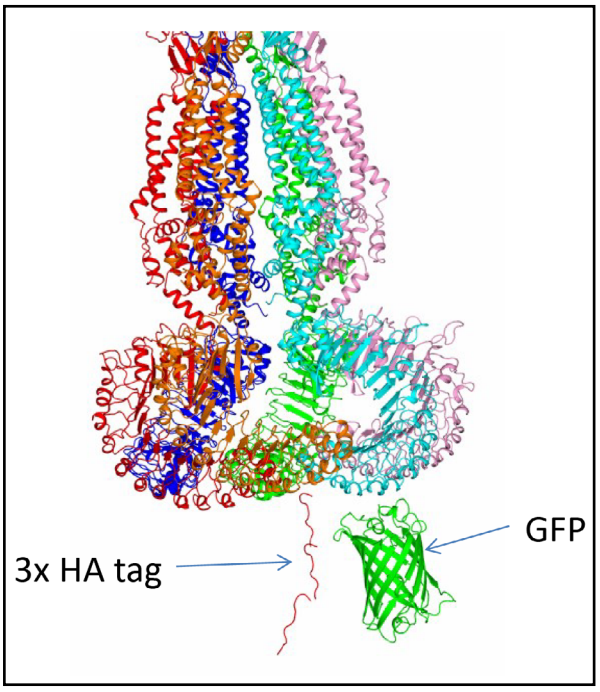

Fig. 3. Relative size of GFP and 3xHA tags compared to LRRC8. The figure illustrates the size differences between a hexameric LRRC8A channel (pdb 6g9l) [12]), a GFP, and a 3xHA tag.

A second line of evidence that LRRD separation or reorientation accompanies channel activation was provided by FRET measurements [60]. König et al. attached FRET donor and acceptor fluorescent proteins to the C-termini in different LRRC8 subunits and measuring FRET in transfected cells in response to hypotonic stimulation they observed a robust decrease of the signal between the donor/acceptor pairs [60].

Is low ionic strength the relevant physical-chemical parameter inducing VRAC activation?

Despite the nomenclature "volume-regulated anion channel", it is clear that volume *per se*, not being an intensive physical parameter, cannot be directly involved in channel activation. Assuming that VRACs are directly activated by a hypotonic shock (and not via interaction with other proteins or phosphorylation, as discussed below), in principle other intensive parameters related to cell volume like membrane tension, concentration of a specific molecule or cytosolic ionic strength (among many other possibilities) have to be considered as relevant parameters [61]. Intuitively, the idea of a mechanical activation of VRAC through membrane stretch might appear as the most reliable hypothesis, given the nature of a channel activated by an increase in cell volume. However, several studies argued that cells have sufficient amounts of extra-plasma membrane in form of invaginations which

may unfold upon hypotonic challenge. Based on these observations, a direct activation of the channel by stretch is unlikely [62, 63]. Importantly, VRAC can indeed be activated by low intracellular ionic strength in isotonic conditions [55, 64-67]. Even recombinant LRRC8 proteins incorporated in lipid droplet bilayers were shown to be activated by low ionic strength [68]. Since in the droplet bilayer system a purified protein had been employed, the latter result demonstrated that low ionic strength directly acts on the channel protein [68]. Since the LRRDs contain numerous positively and negatively charged residues, one hypothesis is that low ionic strength alters the electrostatic interaction of the LRRDs because the charges are less shielded by solvent ions, leading to channel opening.

However, as discussed in detail by Strange et al. [6], it is unlikely that low ionic strength is indeed the physiologically relevant stimulus. First, the reductions of ionic strength necessary to activate VRAC in patch-clamp recordings is much larger than what would be expected from physiological activation by hypotonicity. Second, in whole cell patch clamp recordings in normal ionic strength, even upon application of a hypotonic extracellular solution, the intracellular ion concentrations are practically "clamped" through the patch pipette, even close to the membrane [6] (Bertelli & Pusch, unpublished results). Thus, in these conditions, activation of VRAC occurs without reduction of intracellular ionic strength. Third, VRAC can be activated in a variety of conditions not associated with alteration of ionic strength or extracellular osmolarity. For example application of GTPγS though the patch pipette activates VRAC in chromaffin cells [69]. The potential physiological role of intracellular ionic strength needs to be determined.

Intracellular and extracellular loops are involved in VRAC activation

By extensive mutagenesis and chimeric constructs, Yamada & Strange [16] found that the extracellular loop connecting TMD1 and TMD2 and the intracellular loop (IL) connecting TMD2 and TMD3 are essential for VRAC function. Several chimeric constructs showed changes in inactivation properties, or exhibited constitutive activity. Most interestingly, homomeric functional channels could be obtained by a chimera of LRR8C containing the IL of LRRC8A [16]. The chimera gave rise to a certain degree of constitutive activation, which could be further stimulated by hypotonicity [16], similar to the addition of GFP tags at the C-terminus [9]. Fig. 4 highlights the IL in the structure of homomeric LRRC8A in orange. Yamada & Strange [16] could narrow down the sequence to an essential region of IL sufficient to generate functional homomeric chimeric LRRC8C channels to the stretch comprising residues 182-206 (numbering referring to LRRC8A). Surprisingly, this region is not resolved in any of the homomeric LRRC8A structures [12-15] nor in the structure of homomeric LRRC8D [18] (Fig. 4).

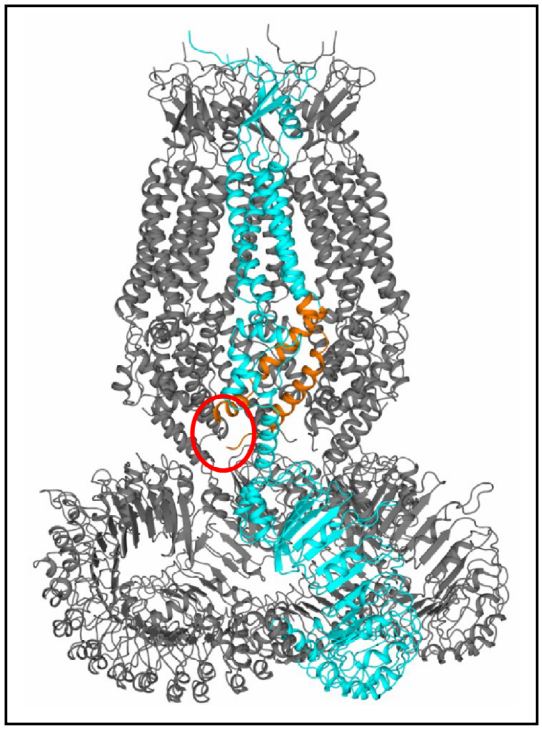

Fig. 4. Mapping of important loops identified by Yamada & Strange. The figure highlights the structural loops identified by Yamada and Strange [16]. One of the 6 subunits is shown in light blue, except the IL in orange. The most essential region is not resolved (highlighted by the red ellipse).

Activation of LRRC8A-LRRC8E heteromers by oxidation

VRAC activity is also dependent on reactive oxygen species (ROS) in several cellular systems [70-72]. In addition, hypotonicity has been reported to lead to increased ROS production [73]. On the other hand, VRAC activation impacts on the oxidation status, probably by the release of reduced glutathione [46]. An important question is whether LRRC8 proteins are directly or indirectly affected by oxidation. Gradogna et al. [58] found that LRRC8 channels are differentially affected by oxidation depending on the subunit composition. While LRRC8A/LRRC8C and LRRC8A/LRRC8D heteromers are inhibited by chloramine-T oxidation, LRRC8A/LRRC8E heteromers are dramatically activated by chloramine-T and tert-butyl hydroperoxide mediated oxidation [58]. In addition, using membrane permeable and impermeable cysteine modifying reagents the authors concluded that oxidation of intracellular cysteines is responsible for the effect. The relevant cysteines have yet to be identified.

Phosphorylation as an essential event for VRAC activation: an open question

Another event that has often been considered among the mechanisms inducing VRAC activation is phosphorylation. Few years after its discovery, in the early 90's, several laboratories spent their efforts to identify subfamilies of kinases and putative upstream pathways involved in the modulation of the channel.

Many studies linked tyrosine phosphorylation and VRAC activation upon swelling, showing that various protein tyrosine kinase (PTKs) inhibitors (like *e.g.* tyrphostin B46, tyrphostin A25 and genistein), when added prior to the hypotonic stimulus, could prevent in a time- and concentration-dependent manner the activation of a current with a biophysical and pharmacological profile of VRAC [74-78]. However, it remained unclear which tyrosine kinase is specifically responsible for the hypotonicity-induced phosphorylation. Lang and collaborators found that the transfection of p56lck, a gene encoding a member of the Src family, involved in the maturation of developing T cells, could restore the osmotic activation of VRAC in lck-deficient lymphocytes [79].

In line with the hypothesis of a PTKs activator effect, it was found that the protein tyrosine phosphatase (PTP) inhibitors, Na_3VO_4 and dephostatin potentiated VRAC when applied after activation of the current by a mild hypotonic shock [76, 77]. Overall, these data suggested a reversible role of tyrosine phosphorylation as a critical step in the regulation of VRAC activity. However, despite the apparent consistency of these conclusions it has to be highlighted that other studies could not detect any effect of such PTK inhibitor compounds on VRAC [80-83]. To make the whole story even more contradictory, other studies reported an increase of VRAC in atrial and ventricular myocytes upon the application of PTK inhibitors [84, 85], and protein tyrosine phosphatase (PTP) inhibitors were shown to suppress VRAC in bovine chromaffin cells and mouse fibroblasts [86, 87].

Even though at first these findings suggested an inhibitory effect of PTK on VRAC, further experiments in myocytes unveiled an antagonistic mechanism of two distinct PTK families, with Src and EGFR kinases showing inhibitory or activating effects, respectively [85]. It was concluded that an interplay of PTKs and their corresponding signaling cascades differentially modulates the gating of the channel in ventricular myocytes. Table 1 summarizes kinase effects and the respective cellular background investigated.

Several hypotheses to justify the difficulty in determining a unique effect of the phosphorylation state of tyrosine residues on VRAC activity have been proposed so far. First, it was suggested that regulatory processes may be connected to tissue- or species-specific mechanisms [62]. Also, broad-spectrum PTK inhibitors, as the most commonly used genistein, can mask antagonistic effects of specific PTKs subfamilies, given that in the very same cell line divergent effect were observed [85]. It has to be stressed that all the above

Table 1. Summary of PTKs and PKCs effects on VRAC activity in different cell types. * = Results observed in the same cell line; # = divergent effects of 2 sub-members of the same kinase family

	Cell subtype	Activation	Inhibition	No effect	Subfamily
PTK	mouse fibroblasts	[74]*	[87]*		
	dog atrial myocytes	[75, 76]			
	human Intestine 407 cell line	[76]			
	calf pulmonary artery endothelial (CPAE) cells	[77]		[81]	
	human atrial and ventricular myocytes	[84, 85]#	[84, 85]#		Src ↑ EGFR ↓
	human T lymphocytes	[79]			Src
	rat osteoblast like cells (ROS)			[80]	
	bovine chromaffin cells		[86]		
PKC	HEK and HELA cells	PKCα [93] (PKD) [60]			
	HT-3 cells	PKCα [91]			
	cardiomyocytes	[92]			
	M-1 mouse cortical collecting duct (CCD) cells	[90]			
	calf pulmonary artery endothelial (CPAE)	[109]		[81]	ROK [109]
	rat C6 glioma cells			[82]	

studies were performed before the discovery of the heteromeric nature of VRAC channels and thus, one possible explanation of divergent results may partially rely on different cell-type specific channel composition and stoichiometry.

A recent important contribution came from the group of Stauber, who used a FRET sensor to monitor VRAC activation. They proposed protein kinase D (PKD) activity as a possible candidate that is critically involved in hypotonicity-induced activation of VRAC [60]. This kinase converts transient diacylglycerol (DAG) signals into physiological effects downstream of PKC, suggesting that serine/threonine phosphorylation may be the final event for VRAC regulation.

There is a large body of literature on a primary role of PKC in VRAC activation. Here, we can only highlight a selected number of contributions. As a general remark it has to be reminded that the use of different drugs to interfere with PKC signaling may lead to different observations as shown by Mongin and colleagues [88, 89]. Application of PKC blockers was able to prevent channel activation under hypotonic conditions, which could be rescued by the PKC activator phorbol 12-myristate,13-acetate (PMA) [90-93]. In particular, two independent studies provided a direct indication of a swelling-induced translocation of PKC-α in the membrane/cytoskeleton fraction and its subsequent phosphorylation in the cell membrane [91, 93]. Furthermore, experiments with a cell line stably expressing kinase dominant negative PKC variants suggested an essential role of PKCα isoforms in swelling-dependent VRAC activation [93].

The molecular identification and the recent determination of VRAC structures may provide a solid tool to revisit previous finding, also taking advantage of the availability of cell lines depleted of endogenous LRRC8 genes. This may help in addressing unsolved questions, on top of which is whether phosphorylation constitutes an obligatory part of swelling induced VRAC activation. Indeed, phosphorylation is one of the first reactions to swelling [22, 76, 94]. However, it remains to be established whether the target of phosphorylation is VRAC itself or another regulatory protein [95]. Structure function studies could be key to uncover residues that may be the final target where single, or different signaling pathways may convey.

Few years before the molecular identity of VRAC was known, in a pioneering work, Abascal & Zardoya not only correctly predicted LRRC8 proteins to form hexameric ion channels, but highlighted also the importance of the intracellular loop connecting the TM pore to the LRR domain as a potential target of post-translational modification [11]. In particular, given the abundance of serine, threonine and tyrosine amino acids in this region and taking advantage of high-throughput proteomic studies, they speculated that such residues in the intracellular loop may undergo phosphorylation.

Recently, as already mentioned above, the group of Strange identified a sequence of the first intracellular loop of the LRRC8A subunit (8A IL), as essential for channel functioning [16]. Their data show that the homomeric chimera of 8C was still functional after the intracellular loop was replaced with residues from 8A IL. Most interestingly, a short minimal stretch of 8A IL was sufficient to give rise to functional homomeric chimeric LRRC8D(8A IL) and LRRC8E(8A IL) as well. From a PONDR analysis (Predictor of Natural Disordered Regions), Yamada & Strange uncovered that this region of the IL, which is not resolved in any of the structures described so far, significantly diverges among LRRC8A, LRRC8C, LRRC8D, and LRRC8E proteins [16]. The authors concluded that such dips in the PONDR score of intrinsically disordered protein regions may predict the existence of molecular recognition elements or other features [6]. In any case, even assuming that such post-translational modifications may occur, it should be assessed whether they are necessary for channel gating, or if they may be required for folding, membrane targeting, protein-protein interaction and/or other physiological roles.

Role of GTPγS in VRAC modulation

The role of GTPγS was deeply investigated by several groups. Application of the non-hydrolysable GTP analogue guanosine 5'-O-(3-thiotriphosphate) into the cell pipette was shown to induce activation of VRAC even in the absence of swelling in several cells type [77, 83, 96]. On the contrary, application of GDPβS, a non-hydrolysable analogue of GDP that competes with GTP for the nucleotide binding sites on G proteins, caused a time-dependent inhibition of VRAC, which was more pronounced when the current was activated by mild hypotonicity. Moreover, some studies reported that treatment of cells with recombinant Clostridium Botulinum or Clostridium Difficile, which catalyzes the UDP-glycosylation of the Rho subfamily of monomeric G proteins, resulted in a strong reduction of osmo-sensitive Cl^- current efflux [97, 98]. These data suggest a role of GTP binding proteins, and specifically of the Rho pathway in the signal transduction leading to VRAC activation. Given that activation of the Ras-related GTPase p21rho was accompanied by a transient reorganization of the F-actin cytoskeleton, Tilly and coworkers [97] proposed an involvement of a Rho signaling cascade and the subsequent rearrangement of actin filaments in the activation of VRAC.

Nevertheless, transfection of calf pulmonary artery endothelial cells with constitutively active isoforms of Gα (a Rho activating heterotrimeric G protein subunit), Rho, or Rho kinase alone failed to induce VRAC activation [98]. Furthermore, GTPγS alone has no effect when applied in Xenopus oocytes in isotonic conditions [99], and, the application of GDPβS could not prevent activation of anion currents in retinal pigment epithelial cells [100]. Altogether, these results make it unlikely that GTPγS signaling has an essential role in VRAC activation and weighs in favor of a permissive or cell subtype specific role rather than an obligatory activation path upon hypotonic stimulation.

Role of intracellular calcium in VRAC activation

The role of intracellular calcium, $[Ca^{2+}]_{int}$, in the activation of VRAC remains rather enigmatic. $[Ca^{2+}]_{int}$ impacts the function of many enzymes, and therefore it is difficult to distinguish direct from indirect effects on VRAC [91, 92, 101]. In several systems, exposure

of cells to hypotonic conditions leads to a rise of $[Ca^{2+}]_{int}$ [62]. However, unlike Ca^{2+}-activated Cl^- channels (*e.g.* bestrophin and TMEM16A), whose activation require a rise in $[Ca^{2+}]_{int}$ as a fundamental for activation, canonical volume-regulated Cl^- channels directly activate in response to swelling, and no direct evidence of a compulsory role of intracellular Ca^{2+} has been reported so far [24, 102, 103].

It is generally assumed that a non-zero baseline concentration of $[Ca^{2+}]_{int}$ is necessary for VRAC activation [104]. Nevertheless, at least in some systems, VRAC can be activated even under conditions of heavy buffering of $[Ca^{2+}]_{int}$ [90, 105], suggesting that $[Ca^{2+}]_{int}$ is not a primary parameter determining VRAC activity.

In our unpublished whole cell experiments in HEK293 LRRC8 KO cells transfected with LRRC8A and LRRC8E, using a pipette solution with 1 μM free Ca^{2+} resulted in a slight but only transient activation of VRAC currents in isotonic conditions. In agreement with the above cited results [90, 105]. In another set of experiments, using an intracellular solution without Ca^{2+} and heavy buffering did not prevent VRAC activation upon hypotonic perfusion (data not shown).

Dependence of VRAC on intracellular ATP

It has been reported in several cell types that activation of VRAC requires a minimal presence of intracellular ATP [90, 106, 107]. As reviewed in detail by Okada's group, ATP acts in a non-hydrolytic manner and presumably binds directly to VRAC channels [62, 108]. However, the relevant ATP binding site has yet to be identified and it cannot be excluded that ATP is acting indirectly on other essential proteins.

Concluding remarks

Surprisingly, even after decades of study, and almost six years after the availability of molecular structures of VRAC, the essential molecular mechanism underlying the activation by hypotonicity and other triggers remains largely unknown. Putting it the other way round – the investigation of these mechanisms remain an important and interesting aspect of fundamental research with a significant relevance in several pathological situations involving VRAC channel activity.

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Author Contributions

S.B. and M.P. prepared the first draft of the manuscript, all authors contributed to finalizing the manuscript and to the preparation of figures.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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