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INVITED REVIEW

VRAC: molecular identification as LRRC8 heteromers with differential functions

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Abstract A major player of vertebrate cell volume regulation is the volume-regulated anion channel (VRAC), which conducts halide ions and organic osmolytes to counteract osmotic imbalances. The molecular entity of this channel was unknown until very recently, although its biophysical characteristics and diverse physiological roles have been extensively studied over the last 30 years. On the road to the molecular identification of VRAC, experimental difficulties led to the proposal of a variety of false candidates. In 2014, in a final breakthrough, two groups independently identified LRRC8A as indispensable component of VRAC. LRRC8A is part of the leucine-rich repeat containing 8 family, which is comprised of five members (LRRC8A-E). Of those, LRRC8A is an obligatory subunit of VRAC but it needs at least one of the other family members to mediate the swelling-induced Cl^- current $I_{Cl,vol}$. This review discusses the remarkable journey which led to the molecular identification of VRAC, evidence for LRRC8 proteins forming the VRAC pore and their heteromeric assembly. Furthermore, first major insights on the role of LRRC8 proteins in cancer drug resistance and apoptosis and the role of LRRC8D in cisplatin and taurine transport will be summarized.

Keywords Chloride channel · Swelling-activated · VSOAC · VSOR

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Introduction

By the end of the last decade, the majority of well-characterized ionic currents across the plasma membrane had been ascribed to specific molecular entities. One remarkable exception was the volume-regulated anion channel (VRAC) which eluded identification for almost 30 years after it had been first described functionally in the 1980s [21, 24, 26].

Several chloride channels are directly sensitive to changes in cell volume, including members of the bestrophin [13] and CLC families [22], while others, like anoctamins, can respond indirectly, e.g., through changes in $[Ca^{2+}]_i$ [33]. However, VRAC specifically refers to a channel mediating the ubiquitous volume-activated chloride current ($I_{Cl,vol}$ or $I_{Cl,swell}$). Characteristics which define $I_{Cl,vol}$ and distinguish it from other anion currents include its virtual absence under isotonic conditions, slow activation in response to hypotonicity or decreases in intracellular ionic strength [12, 66], mild outward rectification, iodide over chloride selectivity, and pronounced inactivation at inside-positive potentials [44]. Several mechanisms for the activation of the current have been proposed, including oxidative processes, changes in intracellular crowding, membrane or cytoskeleton stretch, phosphorylation, and other signaling pathways. Physiologically, $I_{Cl,vol}$ was implicated in regulatory volume decrease (RVD), which reduces cellular volume after acute swelling in response to hypotonic challenges. RVD is achieved by the loss of intracellular solutes, mainly K^+ and Cl^-, as well as osmotically active organic molecules like taurine, glutamate, and myo-inositol, which then drives an outflow of water (Fig. 1). VRAC was proposed to mediate both, anion and osmolyte fluxes; hence, the alternative label volume-sensitive organic osmolyte/anion channel (VSOAC) [32]. However, several studies found divergent regulatory and pharmacological

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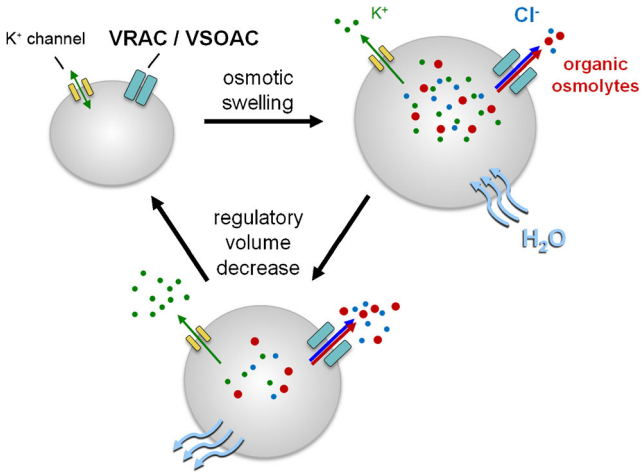

Fig. 1 Schematic representation of the role of VRAC in regulatory volume decrease. Cells swell upon hypotonic challenges, which leads to an opening of VRAC channels that entails an efflux of Cl^- and organic osmolytes. In cases where Cl^- is the main transport substrate, VRAC needs the efflux of potassium through K^+ channels for electroneutrality. Although several K^+ channels can be stimulated by cell swelling, a large constitutive K^+ conductance (as shown) may be sufficient for this purpose. The efflux of osmotically active substances drives water efflux across the plasma membrane, either by diffusion through the lipid bilayer or, more efficiently, through dedicated water channels (aquaporins, not shown). This allows the cellular volume to decrease to normal levels

properties of those two transport activities, arguing for separate pathways and fueling considerable controversy [57].

Since VRAC stubbornly evaded identification, evidence for the association between RVD and $I_{Cl,vol}$ came solely from rather unspecific pharmacological inhibition. The same applied to other suggested physiological roles of VRAC, including its involvement in apoptosis, cell migration, and cell cycle maintenance [27, 44, 47]. Thus, the search for VRAC remained one of the most luring endeavors in the field of ion channel research.

The long search for VRAC—obstacles on the route to its identification

Several factors precluded the molecular identification of VRAC for over 20 years. First, the ubiquitous presence of the characteristic $I_{Cl,vol}$ current in all vertebrate cell types investigated thus far hampered the use of expression cloning, a technique that had been successfully used in the identification of several other ion channels [8, 14, 56]. Endogenous VRAC currents also severely disturb the validation of heterologously expressed VRAC candidate proteins. In fact, heterologous expression of two structurally completely unrelated proteins has been reported to elicit the same characteristic, endogenous current [7]. Hence non-transfected control cells do not necessarily solve the problem of endogenous background currents. Background-free expression systems are needed for unambiguous tests of putative ion channel proteins.

Another commonly used method for the identification of proteins employs affinity purification with a highly specific ligand or inhibitor. This method has been used to successfully identify several ion channel-forming proteins [5, 20]. However, all known VRAC inhibitors also target other proteins, including ion channels and transporters, at similar concentrations.

Another very important obstacle for the molecular identification of VRAC, which became evident only in retrospect, is its heteromeric composition. Since the channel is not comprised by a single molecular entity, but consists of an essential subunit (LRRC8A) and one or more copies of one of the other LRRC8 family members [67], assays using heterologous expression of single proteins were doomed to fail. In the possible scenario that these heteromers instead of containing an obligatory subunit (LRRC8A), would consist of exchangeable subunits, even an siRNA loss of function screen would have been a priori unsuccessful. Since we now know that even heterologous over-expression of LRRC8A together with other LRRC8 subunits did not increase $I_{Cl,vol}$ amplitudes above wild type levels [67], probably every cloning attempt by heterologous expression was destined to fail.

During the past decades, however, a large number of reports suggested several different proteins to form VRAC or to be part of it. These "candidates," all of which proved to be wrong, have been reviewed recently [49]. Here, we will only focus on the three most prominent examples. One of the first proteins suggested to embody VRAC was the P-glycoprotein (P-gp), encoded by the multidrug resistance 1 (*MDR1*) gene in humans [63]. Because of its homology to the chloride channel CFTR, the idea of P-gp forming a channel seemed appealing. Overexpression of P-gp in NIH 3T3 fibroblasts led to a volume-sensitive chloride current that appeared to be absent in untransfected control cells [63]. However, these cells were later shown to display the endogenous $I_{Cl,vol}$ current [23, 43]. P-gp was finally discarded as VRAC candidate by numerous studies involving mutant versions of the protein, antisense oligonucleotide experiments, and expression in *Xenopus laevis* oocytes [18, 42, 62, 64]. A second prominent candidate came from an expression cloning attempt in *Xenopus laevis* oocytes and was termed pI_{Cln} [48]. However, it later turned out to be a spliceosome component [52]. A member of the CLC family of chloride channels and transporters has also been implicated in VRAC function. ClC-3, a Cl^-/H^+ exchanger localized almost exclusively to intracellular compartments, was repeatedly assumed to be VRAC even though several groups showed that $I_{Cl,vol}$ is unchanged in cells from ClC-

3 knockout mice [3, 19, 60]. Most likely all these failures can be explained by the activation of endogenous VRAC currents.

The identification of LRRC8 heteromers as VRAC

In 2014, our group and that of Ardem Patapoutian simultaneously identified LRRC8A as essential component of the mammalian VRAC [53, 67]. Both groups used a genome-wide loss of function screen and hypotonicity-induced quenching of a yellow fluorescent protein (YFP) by iodide as readout. YFP quenching by iodide had already been used at a smaller scale to test candidate proteins in the identification of the calcium-activated chloride channel TMEM16A [9].

Since VRAC is highly permeable for iodide, the iodide-sensitive YFP variant YFP(H148Q/I152L) [15] was introduced into HEK293 cells to generate a stable cell line to be used in a fluorescence-based VRAC activity assay. This approach required the determination of the quenching kinetics after hypotonic stimulation in a high-throughput format. Both groups used the FLIPR® imaging device which permits the simultaneous pipetting and measurement of fluorescence in all wells of a 384-well plate. In their genome-wide siRNA screens, the two groups used different siRNA libraries. In retrospect, this fact reduced the probability that both groups failed to identify an additional important VRAC component due to ineffective siRNAs. The groups performed their assay in slightly different ways. Whereas Qiu et al. opted to pre-swell the cells to open VRAC before adding the iodide solution, our group chose to apply the hypotonic stimulus together with iodide. While the first approach might be beneficial for the identification of the channel protein itself because it minimizes influences of the time course of channel activation by hypotonic swelling, the latter assay allows for the identification of not only the channel protein but also regulatory or signaling processes.

These independent screens revealed that LRRC8A siRNA knockdown reduced swelling-activated iodide influx into the reporter cells. Subsequent electrophysiological studies with cells in which LRRC8A expression was reduced by using siRNA knockdown [53, 67] or completely abolished by genomic disruption using the CRISPR/Cas9 method [67] confirmed that LRRC8A is indispensable for VRAC activity. Upon LRRC8A overexpression, however, both groups noted an unexpected suppression of endogenous $I_{Cl,vol}$ currents instead of an expected large increase in amplitude. This finding hinted at the formation of LRRC8A-containing heteromers, which was further investigated by our group. LRRC8A is a member of the leucine-rich repeat containing family 8 (LRRC8) protein family, which consists of five members LRRC8A-LRRC8E. We decided to thoroughly examine their role in VRAC activity. Knockout cell lines with single deletions of each of the LRRC8B-E proteins revealed that their individual ablation does not eliminate VRAC currents. However, when deleting all four LRRC8B-E proteins, $I_{Cl,vol}$ was abolished (Fig. 2a). Likewise, coexpression of LRRC8A with at least one of the other family members was needed to reconstitute VRAC activity in a *LRRC8* quintuple knockout cell line (Fig. 2a; [67]). A physical interaction between the different LRRC8 members was shown by coimmunoprecipitation [40, 67], and the trafficking of LRRC8B-E to the plasma membrane depended on the coexpression of LRRC8A [67]. Interestingly, expression of different combinations of LRRC8 proteins yielded $I_{Cl,vol}$ currents with strikingly different inactivation kinetics (Fig. 2a, b; [67]). While currents of LRRC8C-containing heteromers generally inactivated more slowly, LRRC8E- and LRRC8D-mediated currents showed fast inactivation at membrane potentials above 60 mV. Similar differences in current inactivation had been previously reported for native $I_{Cl,vol}$ currents [25, 38, 65]. The expression pattern of different LRRC8 isoforms correlated with the inactivation properties of $I_{Cl,vol}$ in the respective tissues and cells [67].

Do LRRC8 proteins form the VRAC pore?

So far, four lines of evidence suggest that LRRC8 proteins are integral parts of VRAC and may form its pore. First, even before the identification of LRRC8 proteins as VRAC components, Abascal and Zardoya [1] detected a significant homology of LRRC8 proteins to pannexins. They therefore suggested that LRRC8 proteins form hexameric channels [1]. The second line of evidence came from strikingly different kinetics of $I_{Cl,vol}$ inactivation observed with different combinations of LRRC8s, as discussed above [67]. As fast current inactivation in other ion channels is generally mediated by either the pore-forming α-subunits (Na_V and K_V channels [10, 28]) or closely associated accessory proteins (e.g., K_V β-subunits [54]) but cannot be explained by a signal transduction cascade, we concluded that LRRC8 proteins must be an integral part of the VRAC channel. However, this observation falls short of proving that they form its pore.

The third line of evidence was based on a cysteine substitution and modification study of LRRC8A, which identified threonine 44 in transmembrane segment 1 (TM1) as a likely pore-lining residue [53]. Modestly altered $I^- > Cl^-$ selectivity of $I_{Cl,vol}$ obtained by overexpression of LRRC8A mutants T44C and T44R in stable *LRRC8A* knockdown cells further hinted at a direct involvement of LRRC8A TM1 in the formation of the pore. However, T44 mutants yielded rather low currents, increasing the contribution of confounding background currents, and the absence of stronger effects of mutations to charged residues on $I^- > Cl^-$ selectivity has met with some criticism [2].

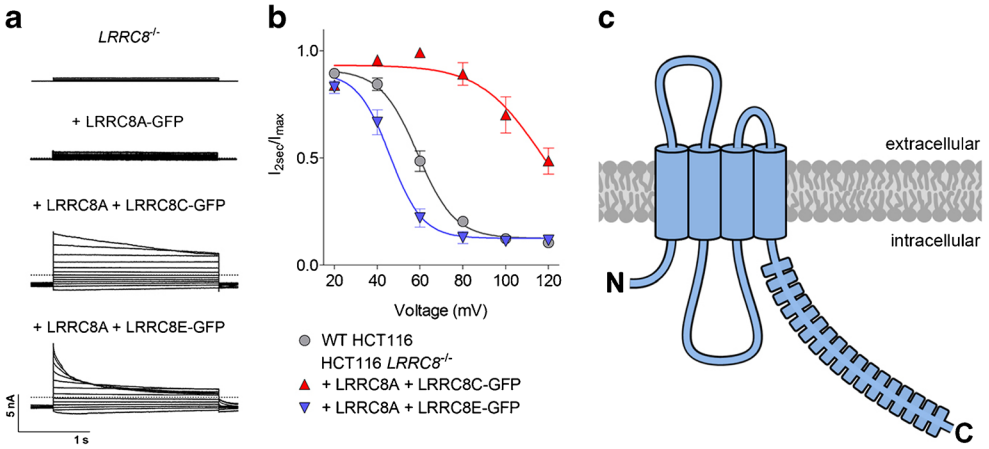

Fig. 2 LRRC8 heteromers are essential components of VRAC. **a** Representative currents from *LRRC8*$^{-/-}$ HCT116 cells (lacking all LRRC8 isoforms) clamped to voltages between −120 and 120 mV in 20 mV increments. Cells were either untransfected (*top*) or transfected with the indicated LRRC8 combinations. While currents were absent in *LRRC8*$^{-/-}$ cells and LRRC8A alone failed to restore $I_{Cl,vol}$, coexpressing LRRC8A with either LRRC8C or LRRC8E led to robust currents that differed drastically in their inactivation kinetics. **b** $I_{Cl,vol}$ inactivation assessed by ratio of current at end and beginning of pulse. **c** Schematic of the transmembrane topology of LRRC8 proteins

Strong evidence for LRRC8 proteins forming the VRAC pore comes from our finding that LRRC8 subunit composition determines the substrate selectivity of VRACs [50]. This was most convincingly demonstrated by the comparison of transport ratios between knockout cell lines with reduced complexity of LRRC8 heteromeric assembly. In line with the previous finding that LRRC8D is required for the uptake of blasticidin S [40], our results indicate a special role for LRRC8D-containing VRACs in the transport of larger substrates, as LRRC8A/LRRC8D heteromers exhibited up to >10-fold higher cisplatin/Cl^- and taurine/Cl^- transport ratios than LRRC8A/LRRC8C heteromers [50].

Membrane topology and multimerization of LRRC8 proteins

Hydrophobicity analysis suggests that LRRC8 proteins contain four transmembrane domains and a large C-terminal tail with up to 17 of the eponymous leucine-rich repeat domains (Fig. 2c). Prior to the identification of LRRC8 proteins as VRAC-forming proteins, their N- and C-termini were suggested to be located on the extracellular side, with the long leucine-rich repeat-containing C-terminus assumed to function as a ligand-binding domain [35, 55]. This was an obvious assumption since these domains form the ligand-binding, extracellular part of the receptor in numerous other leucine-rich repeat containing proteins like toll-like receptors [11, 17]. However, this hypothesis had to be revised since various experimental approaches [40, 53, 67] and bioinformatical analysis of databases [1] established the opposite topology in which both the N- and the C-terminus of LRRC8 proteins are located in the cytosol. This also agrees with the homology of LRRC8 proteins to pannexins [1]. This evolutionary relationship further suggested that LRRC8 proteins might form hexameric complexes like pannexins, which are structurally similar to the well-studied family of gap-junction forming connexins without sharing sequence identity [1].

Role of VRAC in transport of organic osmolytes

The release of organic osmolytes during RVD is an important process in vertebrate cells. It includes the transport of three classes of molecules; polyols (e.g., *myo*-inositol), amino acids, or amino acid derivatives (e.g., aspartate, glutamate, or taurine), and methylamines (e.g., ethanolamine) [4, 31]. Taurine is by far the most thoroughly studied organic osmolyte in RVD because of its high abundance in vertebrate cells (intracellular taurine concentrations range from 5 to 50 mM [36]) and because of its metabolic inertness [61] which allow radiotracer experiments that are not confounded by metabolism. As discussed earlier, there has been an ongoing debate whether VSOAC is identical to VRAC (as excellently reviewed elsewhere [57]). After the recent discovery of LRRC8 heteromers as essential VRAC components [53, 67], it was possible to rigorously address this issue for the first time. Swelling-induced efflux of radiolabeled taurine was abolished in HeLa cells and HEK293 or HCT116 cells, where *LRRC8A* was knocked down using RNAi or knocked out using CRISPR/Cas9-mediated genome editing, respectively [53, 67]. Taurine transport could be partially rescued in *LRRC8A*$^{-/-}$ cells by cotransfection of LRRC8A and LRRC8C [67]. Taurine efflux was also abolished in *LRRC8(B-E)*$^{-/-}$ cells that lack all LRRC8 proteins except LRRC8A [67]. Hence, both $I_{Cl,vol}$ and swelling-induced taurine flux depend on LRRC8 heteromers.

Recently, we showed that knockout of LRRC8D strongly reduced swelling-induced taurine fluxes in HEK293 cells [50]. Furthermore, cells expressing LRRC8A/LRRC8D heteromers yielded taurine fluxes quantitatively similar to those of wild-type cells, whereas taurine efflux was nearly abolished with LRRC8A/C heteromers. Since $I_{Cl,vol}$ amplitudes were at wild-type levels in A/C expressing, but strongly reduced in A/D expressing cells, it was concluded that LRRC8D increases the selectivity of VRAC for taurine. RVD was decreased (but not abolished) in *LRRC8D*$^{-/-}$ cells in spite of unchanged $I_{Cl,vol}$. This revealed an important role of taurine or other organic osmolytes in volume regulation.

Together, these findings show that the previously reported differences between "VRAC" and "VSOAC" may be explained by the presence of differently composed LRRC8 heteromers which have overlapping but distinct permeation properties and may be regulated differently. The wide expression pattern of most LRRC8 isoforms suggests that most cells simultaneously express different VRACs, i.e., differently composed LRRC8 heteromers, in their plasma membranes. After a specific role of LRRC8D for the transport of taurine and cisplatin (see below) has now been established, it is tempting to speculate that other LRRC8 isoforms may increase VRAC's permeability for other substrates. This would be of special interest for pathological conditions like stroke, where VRAC mediates the efflux of the excitotoxic amino acids glutamate and aspartate (for a review, see [41]). While a contribution of the crucial LRRC8A subunit in swelling-induced glutamate efflux from astrocytes has already been established using an RNAi-based knockdown of LRRC8A [29], it remains to be explored whether other LRRC8 isoforms are specifically needed for glutamate transport.

VRAC in drug uptake and apoptosis

Cell volume changes are associated with two different cell death modes. Apoptosis, also known as programmed cell death, is characterized by early persistent shrinkage (named apoptotic volume decrease (AVD)) that is followed by caspase activation, nuclear condensation, and DNA fragmentation. Necrosis is accompanied by cell swelling and eventually leads to cell bursting.

In contrast to the rapid RVD response to osmotically induced cell swelling, AVD develops slowly over a period of several minutes to hours after apoptosis is triggered through either mitochondrion- or death receptor-mediated pathways. AVD is believed to be the consequence of an efflux of K^+ and the activation of an outwardly rectifying Cl^- current with the electrophysiological and pharmacological characteristics of VRAC [37]. In AVD, VRAC is activated in cells that are initially neither swollen nor shrunken [46]. AVD is thought to facilitate the progression of apoptosis, although the exact mechanism remains unclear [59]. This conclusion was mainly based on the effect of channel inhibitors on apoptosis. Several VRAC blockers inhibited both apoptosis-induced cell shrinkage, caspase induction, and cell death [34, 37, 45, 46]. However, all these inhibitors are unspecific. The recent identification of LRRC8 heteromers as essential VRAC components [67] now allowed us to strictly test the role of VRAC in AVD using cell lines with disrupted *LRRC8* genes. Indeed, HCT116 cells in which either the *LRRC8A* or all *LRRC8* genes were disrupted (*LRRC8A*$^{-/-}$ or *LRRC8*$^{-/-}$ cells, respectively) displayed strongly reduced caspase activation after exposure to either staurosporine or the anticancer drug cisplatin, proapoptotic drugs that have been extensively used to study a role of VRAC in apoptosis [50]. Since $I_{Cl,vol}$ is abolished and RVD strongly reduced in *LRRC8A*$^{-/-}$ and *LRRC8*$^{-/-}$ cells, these results are compatible with the notion that VRAC-dependent AVD is an important facilitator of drug-induced apoptosis [50].

The resistance of cells to anticancer drugs like cisplatin, but also other drugs, has been suggested to depend on VRAC-dependent AVD in many studies [39, 51]. When compared with parent cells, the cisplatin resistance of KCP-4 tumor cells correlated with decreased $I_{Cl,vol}$ [30, 39]. Importantly, the sensitivity to the Pt-drug and VRAC activity could be restored in parallel by treatment with histone deacetylase inhibitors [39]. Surprisingly, a recent unbiased genomic screen for carboplatin resistance yielded not only the obligatory VRAC subunit LRRC8A, but even more prominently LRRC8D [50]. Both *LRRC8A*$^-$ and *LRRC8D*$^-$ cells displayed higher resistance to cisplatin and carboplatin. Furthermore, downregulation of LRRC8D expression in ovarian cancer patients treated with the Pt-drug appears to be associated with reduced survival [50], thus suggesting that LRRC8D might also affect Pt-drug responses in cancer patients.

The finding that loss of LRRC8D increased cisplatin resistance was unexpected since this subunit is not required for $I_{Cl,vol}$, and because cell volume regulation was not abolished, but only decreased in *LRRC8D*$^{-/-}$ cells [50]. This questions the notion that VRAC-dependent drug resistance is (only) due to a loss of VRAC- and AVD-dependent apoptosis. Caspase induction depended on LRRC8A and LRRC8D when induced by cisplatin, but only on LRRC8A when triggered by staurosporine. Whereas staurosporine evoked apoptosis independently of tonicity, hypotonic cell swelling significantly enhanced cisplatin-induced caspase activity. This suggested that VRAC may transport cisplatin but not staurosporine, and that transport of cisplatin, like that of taurine or blasticidin S [40], depends on the LRRC8D subunit. Indeed, cellular accumulation of Pt-based drugs in cells of various *LRRC8* genotypes revealed that 50–70 % of long-term cisplatin uptake depends on LRRC8A and LRRC8D. The remaining LRRC8-independent Pt transport is mostly passive diffusion across the plasma membrane [16]. Paralleling the augmented

apoptosis in hypotonic solution, cell swelling also boosted cisplatin transport in a VRAC-dependent manner as it could be blocked by carbenoxolone and was absent in *LRRC8A*$^{-/-}$ and *LRRC8*$^{-/-}$ cells. The precise contribution of LRRC8D in Pt transport was assessed by reducing the complexity of the potentially very heterogeneous VRAC population simultaneously expressed in wild-type cells. Pt accumulation was evaluated in knockout cells expressing the obligatory LRRC8A and only one of the other subunits. LRRC8A/D-expressing cells accumulated almost as much Pt as wild-type cells whereas A/C or A/E heteromers allowed as little cisplatin transport as *LRRC8A*$^{-/-}$ cells. However, the latter heteromers could also transport cisplatin to some degree after activation by hypotonic swelling. Finally, we showed that cisplatin slowly activated cellular iodide influx in a LRRC8A-dependent manner, confirming previous suggestions that VRACs are activated in the course of apoptosis [58].

In summary, these results suggest that VRAC plays a dual role in cisplatin resistance (Fig. 3). It enhances the progression of apoptosis, possibly by promoting AVD, but has an additional role in cisplatin/carboplatin uptake. This uptake occurs predominantly through LRRC8A/D-containing channels. As VRAC is activated by proapoptotic stimuli, cisplatin enhances its own uptake in a feed-forward mechanism. It is believed that Pt-based drugs cause apoptosis only at high concentrations that significantly exceed therapeutic levels [6]. Therefore, in clinical settings, VRACs probably influence tumor sensitivity mainly by mediating the uptake of Pt-based drugs.

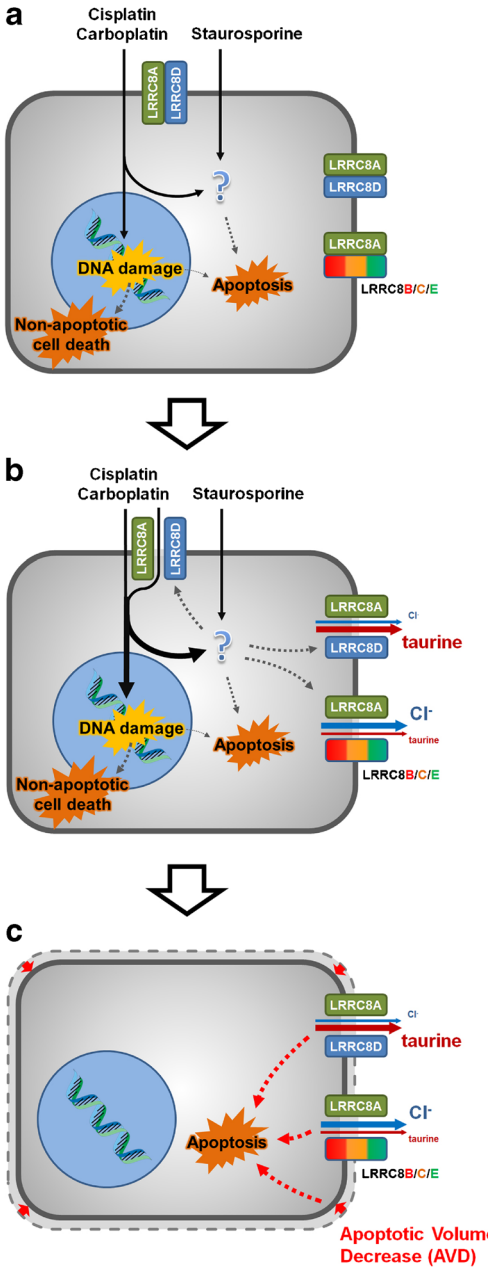

Fig. 3 Dual role of VRAC in drug uptake and apoptosis. **a** Anticancer drugs cisplatin and carboplatin and the proapoptotic staurosporine enter cells through passive diffusion. Pt-based drugs induce nonapoptotic cell death, and to a lesser extent, apoptosis through DNA damage, while both Pt-based drugs and staurosporine can induce apoptosis through an additional, poorly characterized pathway. **b** VRACs are also activated by proapoptotic stimuli. VRAC channels mainly transport osmolytes but also provide a further pathway for uptake of Pt-based drugs, but not for the larger staurosporine. Incorporation of the LRRC8D subunit largely increases VRAC's ability to transport taurine and cisplatin. **c** Cl^- and osmolyte efflux through drug-activated VRACs trigger AVD, which facilitates the progression of apoptosis

Outlook

The identification of LRRC8 heteromers as VRAC components now provides a firm basis for clarifying many burning questions in the field of cell volume regulation. This breakthrough already allowed us to understand why different tissues express VRACs with different inactivation kinetics and suggests that the previously observed differences between VRAC and VSOAC result from the formation of differently composed LRRC8 heteromers with different properties. It also led to the unexpected discovery that VRAC transports anticancer drugs and that this channel has, together with its role in apoptosis, a dual role in drug resistance. Future studies will certainly address VRAC's structure and its relationship to function and will further clarify the roles of individual subunits in permeation and channel regulation. The observation that $I_{Cl,vol}$ amplitudes could not be increased by overexpression of several combinations of LRRC8 isoforms is vexing and may point to the existence of other VRAC components. By working backward from VRAC it should be possible to better understand the signal transduction cascade involved in cell volume regulation. Using genetically modified mice, it is now possible to explore the probably numerous physiological functions of VRAC which likely

transcend its role in cell volume regulation. A constitutive *Lrrc8a*$^{-/-}$ mouse [35] already indicates defects in a variety of organs. We expect many surprises.

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