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INVITED REVIEW

Biophysics and Physiology of the Volume-Regulated Anion Channel (VRAC)/Volume-Sensitive Outwardly Rectifying Anion Channel (VSOR)

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Abstract The volume-regulated anion channel (VRAC), also known as the volume-sensitive outwardly rectifying (VSOR) anion channel or the volume-sensitive organic osmolyte/anion channel (VSOAC), is essential for cell volume regulation after swelling in most vertebrate cell types studied to date. In addition to its role in cell volume homeostasis, VRAC has been implicated in numerous other physiological and pathophysiological processes, including cancer, ischemic brain edema, cell motility, proliferation, angiogenesis, programmed cell death, and excitotoxic glutamate release. Although VRAC has been extensively biophysically, pharmacologically, and functionally characterized, its molecular identity was highly controversial until the recent identification of the leucine-rich repeats containing 8A (LRRC8A) protein as essential for the VRAC current in multiple cell types and a likely pore-forming subunit of VRAC. Members of this distantly pannexin-1-related protein family form heteromers, and in addition to LRRC8A, at least another LRRC8 family member is required for the formation of a functional VRAC. This review summarizes the biophysical and pharmacological properties of VRAC, highlights its main physiological functions and pathophysiological implications, and outlines the search for its molecular identity.

Keywords Cell swelling · Cell volume · LRRC8A · Osmotic · RVD · VRAC · VSOR · VSOAC

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Introduction

The mechanisms controlling cell volume are essential for normal cell function. Their importance extends far beyond cell volume homeostasis, as regulated cell volume changes are involved in numerous physiological processes, and conversely, cell volume perturbation is associated with a range of pathological states. After cell swelling, volume is recovered in the process of regulatory volume decrease (RVD), a major component of which is activation of an anion current. Historically, the channel mediating this current is known as the volume-regulated anion channel (VRAC), the volume-sensitive outwardly rectifying (VSOR) anion channel, or the volume-sensitive organic osmolyte/anion channel (VSOAC). For the sake of consistency with the recently published papers on LRRC8, we refer in the following to the channel as VRAC. The recent identification of leucine-rich repeats containing 8A (LRRC8A) as essential for VRAC currents and likely part of the channel pore (see review by Jentsch and coworkers elsewhere in this volume) opens up for novel understanding of the regulation and roles of this previously so elusive channel. It is therefore timely–and the purpose of this review–to recapitulate briefly the discovery of VRAC, its biophysical properties, pharmacology and regulation, and its physiological and pathophysiological roles.

The discovery of the VRAC current as an essential part of the RVD response

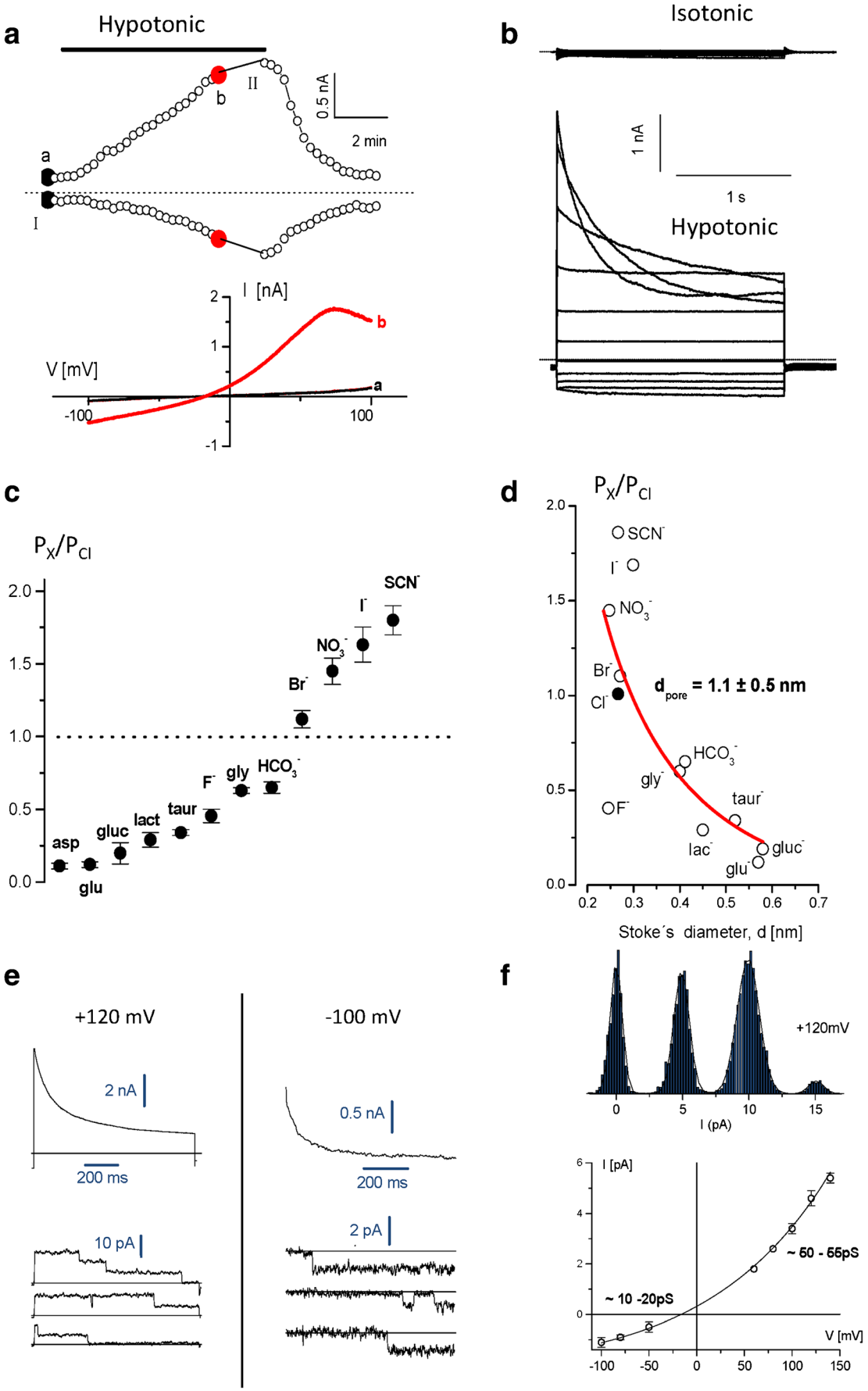
The discovery that osmotic swelling increases cellular anion (and cation) permeability was first made in Ehrlich ascites tumor cells [1–3] and human lymphocytes [4–6], followed by electrophysiological recording of an outwardly rectifying Cl^- current after hypotonic swelling of human T lymphocytes and intestine 407 human epithelial cells [7, 8]. Shortly thereafter, the first comprehensive biophysical analysis of VRAC was published [9], as further detailed below and in Fig. 1. Moreover, this current was shown to be necessary for the RVD response [7, 8].

The search for the molecular identity of VRAC

As discussed elsewhere, several properties of VRAC contributed to its long resistance to discovery at the molecular level [11, 12]. The colorful history of the search for VRAC involves numerous now-discarded molecular candidates (reviewed in [11–18]). The first proposed candidates were P-glycoprotein (P-gp, aka multidrug resistance protein-1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1)) [19], pICln [20], and ClC-3 [21], which were all later shown to exhibit biophysical properties incompatible with those of VRAC and/or to be unnecessary for the VRAC current [10, 16, 22]. Thus, depletion or inhibition of P-gp had no effect on VRAC in intestine 407 cells [23] and VRAC current and P-gp expression do not correlate across cell types [24]; ClC-3 knockout mice exhibit normal VRAC currents [25–27]; and pICln is a protein involved in spliceosomal snRNP biogenesis [28, 29]. Furthermore, pICln, when expressed in lipid bilayers, has been shown to form highly cation-selective channels [30], although ion selectivity was later proposed to reflect the specific lipid composition of the bilayer [31]. Additional proteins proposed as VRAC include band 3, aka the AE1 Cl^-/HCO_3^- exchanger [32]; the FXYD family protein phospholemman [33–35]; the voltage-dependent mitochondrial anion channel VDAC [36]; and the intracellular Cl^- channel CLIC1 [37, 38]. Evidence against all of these as molecular candidates for VRAC has later been brought forward (see [10, 13, 15, 16, 22]). In addition to LRRC8A, two other candidates have been brought forward in recent years. Firstly, TMEM16A [39] was proposed as a VRAC candidate; however, its Ca^{2+} sensitivity clearly distinguishes it from VRAC. Also knockdown of several other TMEM16 family members (TMEM16F, −H, and −J) was found to reduce VRAC [39]. Later studies showed that TMEM16F does not mediate VRAC, yet it likely contributes to RVD under conditions with increased free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [40, 41] (for a discussion of the variable role of Ca^{2+} in RVD, see [14]). Notably, TMEM16F is an ATP-independent phospholipid scramblase, and recent work has proposed that the ionic current is a nonspecific leak current resulting from the scramblase activity [42]. On the other hand, recent evidence suggests that members of the TMEM16 family, at least, under some conditions induce VRAC currents and, moreover, may engage in a functional relationship with LRRC8A [43, 44].

Finally, bestrophin has been described as the molecular entity underlying VRAC in *Drosophila melanogaster*, first by Chien and Hartzell [45] and later independently verified in a genome-wide siRNA screen [46]. This suggests, interestingly, a divergence of volume-sensitive Cl^- channels between invertebrates and vertebrates in the course of evolution. The possible role of bestrophin in mediating VRAC currents in vertebrates is controversial. VRAC is unaffected in macrophages from mBest1/2 double knockout mice [47], and the biophysical properties of VRAC in mammalian cells are clearly at variance with those of the *Drosophila* volume-sensitive Cl^- current [46]. On the other hand, a recent report found the mammalian bestrophin 1 (*BEST1*) protein to be essential for RVD in human retinal pigment epithelial cells and to mediate

Fig. 1 Biophysical properties of VRAC. **a** Time course of the activation and deactivation of currents through VRAC in a mouse aorta endothelial cell (measured at +80 and −80 mV). The *solid bar* marks the application of a 25 % hypotonic solution (*upper panel*). Note the slow activation of VRAC. Current–voltage (*I–V*) curves (*lower panel*) obtained at the time points indicated by *a* and *b* in the upper panel. **b** Time dependent current–voltage relationship. The *upper panel* shows current traces under isotonic conditions taken at the time indicated by *I* in panel (**a**). The *lower panel* shows current traces during a hypotonic challenge (indicated by *II* in panel (**a**)). Traces are from a step-voltage protocol (holding potential 0 mV, 2 s steps from −80 to +100 mV, spaced 20 mV). Note the inactivation at positive potentials. **c** Permeation properties of VRAC: permeability ratios calculated from the shifts in E_{rev} by substitution of Cl^- (*asp* aspartate, *glu* glutamate, *gluc* gluconate, *lact* lactate, *taur* taurine, *gly* glycine, HCO_3^- bicarbonate, SCN^- thiocyanate). Note the permeation of large anion such as taurine, aspartate, and glutamate. **d** Stoke's diameters of the anions used for Cl^- substitution plotted against their permeability relative to Cl^-. The diameter of the open VRAC pore was estimated from the excluded volume model (*solid line*). **e** Single channel properties of VRAC. Ensemble-averaged membrane currents in a BC3H1 myoblast cell after a 40 % decrease in extracellular osmolarity, recorded after voltage steps from −80 to + 120 mV (*upper panel*, *left*). *Bottom left*: single VRAC currents from an outside-out patch obtained from the same cell and using the same voltage protocol. Note the clustering of openings at the beginning of the step indicating a decrease in open probability during the step. *Top right*: current-trace (ensemble-averaged current) for a step from +80 to 100 mV. Note the slow increase in current, reflecting recovery from inactivation at the positive holding potential. *Bottom right*: outside-out patch, single channel recordings from the same cell as above. Note the delay in the appearance of channel openings, which parallels the slow increase in the macroscopic inward current. **f** Amplitude histograms from single channel openings after cell swelling (outside-out patch pulled from a swollen cell held at −80 mV and stepped to +120 mV). The histogram was fitted by four Gaussian functions. Single channel amplitude was 5.0 pA. Plotted below is the entire single channel current–voltage relationship from these experiments (BC3H1 cells). Note that the outward rectification is due to the different slope conductance at positive and negative potentials. Panels **a**–**f** are modified, with permission from Figures 13–15 in [10]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10 pA
0.5 nA
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+120mV
I (pA)
I [pA]
~ 50 - 65pS
~ 10 -20pS
V [mV]

currents in these cells with biophysical properties comparable to those of VRAC [48]. It may however be noted that the ohmic current–voltage relationship and essentially absent voltage-dependent inactivation are at variance with those of VRAC (compare Fig. 4 in [48] with Fig. 1 of this review). Notably, it was shown in this study that LRRC8A was dispensable for both VRAC and RVD [48].

A major breakthrough came, as noted above, simultaneously from two groups in 2014: the Jentsch group [59] and the Patapoutian group [60], who both identified the LRRC8 family as essential for VRAC. This work will be covered elsewhere in this volume, and only the major conclusions are briefly recapitulated here. Functional VRAC currents appear to require LRRC8A and at least one other LRRC8 isoform. Further, although the structure-function characterization of the assumed LRRC8 hexamer (or other multimers) is still very incomplete and some contradictory evidence is obtained (see [56]), it was proposed based on mutagenesis studies that LRRC8A may form part of the pore or be located very close to it [59, 60]. Specifically, it was suggested that residue T44 of human LRRC8A, which is predicted to localize in the external part of TM1, forms part of the channel pore [60]. The recent report that the subunit composition of the LRRC8 heteromer determines its permeability properties is also most consistent with the LRRC8 proteins contributing directly to the pore [52, 61]. On the other hand, the fact that mutations of charged amino acids in predicted transmembrane domains have little effect on the current [60] is surprising if these comprise part of the pore [56]. Also consistent with its role as VRAC, LRRC8A is very broadly expressed, localizes to the plasma membrane, is, like VRAC (see below, and [62–64]), isovolumetrically activated by reduced intracellular ionic strength [60], and, finally, its knockdown inhibits RVD [59, 60]. Finally, an important open question, which can now begin to be addressed, is whether LRRC8A may in fact have roles beyond its function as an ion channel or whether the phenotype of the LRRC8A knockout mouse [65] and that of a patient with a truncated LRRC8A variant [66] reflects novel VRAC functions.

VRAC biophysical and permeation properties

Current characteristics

The biophysical characteristics of VRAC have been described in detail in a wide range of cell types (e.g. [9, 16, 17, 22, 56, 67]; for reviews, see e.g. [10, 11, 17, 22, 49, 56, 68]). The VRAC current activates slowly when cells are exposed to a hypotonic challenge. It exhibits a modest outward rectification which is known to reflect voltage-dependent enhancement of the single channel conductance [16, 68, 69] (Fig. 1a, b). The single channel conductance is in the intermediate conductance range, approximately 50–80 pS at positive, and 10–20 pS at negative membrane potentials [69–74] (Fig. 1e, f). It may be noted that the single channel current was initially greatly underestimated due to incompatibility of the current activation properties with the assumptions of stationary noise analysis [70, 71]. Specifically, Jackson and Strange demonstrated that activation of the current by swelling may involve a sudden switching of single channels from a closed state, where channel open probability is zero, to a state, and where open probability is near unity [70, 71]. The current generally exhibits a characteristic, but variable, voltage-dependent inactivation at positive membrane potentials, the time course of which is sensitive to extracellular concentrations of H^+, Mg^{2+}, and Cl^-, as well as on the current magnitude [9, 16, 22, 56, 67, 75] (Fig. 1b). Similar voltage-dependent inactivation is observed at the whole cell and single channel level [69, 75] (Fig. 1b, e).

Permeability profile

The VRAC permeability sequence has been characterized in detail and is generally reported as $SCN^- > I^- > NO_3^- > Br^- > Cl^- > HCO_3^- >$ glycine $> F^-$ > taurine > lactate > gluconate > glutamate > aspartate [9, 10, 16, 22, 76–78] (Fig. 1c). A fit of the relative permeabilities of these ions to their Stoke's diameter predicts a pore diameter of about 11 Å (Fig. 1d). The pore geometry was also more precisely estimated by use of 4 sulfonic-calix(n)arene anions as permeation reporters indicating that calix(4)arene permeates but calix(6)arene blocks the VRAC pore, indicating an 11 by 17 Å pore [79, 80]. Non-electrolyte partition studies pointed to a cut-off diameter of the VRAC pore of 12.6 Å [81].

The permeability of VRAC to organic anions, leading to its naming also as VSOAC (volume-sensitive osmolyte anion channel), has been widely studied and whether inorganic and organic anions permeated the same or different channels has been subject to major controversy (that may in fact reflect the involvement of partially different LRRC8 heteromers, see below); for the earliest evidence of organic anion transport via VRAC, see [82]; for discussions of this topic, see [14, 83, 84].VRAC has also been proposed to be permeable to the ATP anion [85–87] yet is inhibited by ATP via open-channel block under physiological voltage conditions [17, 88]. Furthermore, VRAC was recently found to potentiate the uptake of the protein synthesis inhibitor blasticidin S [89] and, as further described below, the chemotherapeutic platinum drugs cisplatin and carboplatin (but not oxaliplatin) [52] into mammalian cells. The discovery that LRRC8 channel subunit composition determines its permeability profile [52] suggests that the permeability to both anions and large organic molecules of different charge may reflect the expression of VRAC channels of varying stoichiometry. Thus, the LRRC8A/D heteromer favors permeation of organic osmolyte over anions and also

allows uncharged compounds such as platinum drugs to pass the channel. In contrast, the LRRC8A + B/C/E heteromers favor anion permeation above taurine permeation (for a discussion, see [52]).

VRAC pharmacology

The pharmacology of VRAC has been extensively described (see [15, 16, 56]). Although no fully selective VRAC inhibitors are yet available, numerous compounds strongly or partially inhibit the VRAC current. Such compounds that are widely used include 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and tamoxifen [15, 16, 56]. The acidic di-aryl-urea NS3728 inhibits VRAC in HEK-293 cells and Ehrlich Lettré (ELA) cells with an IC_{50} value of around 0.4 μM [90, 91] and has gained relatively wide use in recent years. This compound, however, also inhibits Ca^{2+}-activated Cl^- currents [91]. Another apparently specific VRAC inhibitor is 4-(2-butyl-6,7-dichlor-2-cyclopentyl-indan-1-on-5-yl)-oxybutyric acid (DCPIB) shown to specifically inhibit VRAC in the heart and CNS [92–94]. Also serotonin reuptake inhibitors (fluoxetin, i.e., Prozac) [95, 96], antimalarials (mefloquin) [97], anti-estrogen (clomiphene, nafoxidine) [98], and the "T-type Ca^{2+} channel blocker" mibefradil [99] exhibit relatively strong inhibitory effects on VRAC currents. Finally, numerous other compounds, albeit not specific to VRAC, have been shown to inhibit the VRAC current, including pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) [100], suramin [100], phloretin [101], and carbenoxolone [102]. Obviously, the high chemical variability of the VRAC-modulating compounds has impeded the evaluation of specific functional properties of VRAC.

VRAC regulation

The regulation of VRAC has been extensively studied, with a particular focus on understanding the signal of activation upon cell swelling and how this information is transmitted to the channel. A complete picture of the mechanisms regulating VRAC and their possible interrelationship is still missing, and some of the findings may have been compromised by the lack of a molecular candidate and specific pharmacological tools for VRAC. Needless to say, the identification of LRRC8A opens for the investigation of this using state-of-the art tools and will undoubtedly soon lead to leaps in the understanding of VRAC regulation. However, in this context, it is useful to know the major evidence on pathways of VRAC activation and modulation identified so far, and these are therefore summarized in the following.

VRAC activation

As its name implies, VRAC is regulated by cell swelling. However, as recently discussed in detail [11], VRAC is likely activated not by the change in cell volume per se – an *extensive thermodynamic parameter*–but by the accompanying decrease in intracellular ionic strength–an *intensive thermodynamic parameter*. Indeed, a reduction of ionic strength under isosmotic conditions does not cause a change in cell volume but results in activation of VRAC and vice versa; an increased intracellular ionic strength, in the form of a hypertonic intracellular solution, causes a volume increase but inhibits VRAC activation [62, 64]. It has been proposed, in part, based on pioneering experiments in erythrocytes [103], that the relative activities of channels/transporters mediating the swelling-activated efflux of organic and inorganic osmolytes may be determined by the extent of ionic strength change during cell swelling [104, 105]. It is emphasized that ionic strength is clearly not the only mechanism capable of activating VRAC. In addition to its activation by reduced intracellular ionic strength [62–64], VRAC is isovolumetrically activated by intracellular GTPγS [106, 107]. VRAC was also shown to be activated isovolumetrically by purinergic signaling [54, 108], at least in part involving Ca^{2+}-signaling and protein phosphorylation events [109] and via activation of bradykinin (BK) receptors and metabotropic glutamate receptors (mGluR), in a manner involving reactive oxygen species (ROS) and again Ca^{2+} signaling [54–56, 58]. ROS were shown to directly induce VRAC activation in a manner independent of cell swelling [53, 110, 111] and to be involved in activation of VRAC by epidermal growth factor (EGF) signaling [111] and by inducers of apoptosis [53]. Swelling-induced VRAC activation was furthermore shown to involve a swelling-induced interaction between α-actinin-4 (ACTN4) and a cytosolic ABC transporter family member, ABCF2, which prevented the inhibitory action of ABCF2 on VRAC [112]. The inflammatory mediator Sphingosine-1-phosphate (S1P) acting via its G-protein-coupled receptor S1PR1 was also recently reported to activate VRAC isovolumetrically. S1P is generated by sphingosine kinase, which is activated by multiple stimuli, including bacterial lipopolysaccharide (LPS), platelet-derived growth factor (PDGF), tumor necrosis factor alpha (TNFα), thrombin, IgE-bound antigen, and ATP [86, 113].

Intracellular signaling pathways in VRAC activation and modulation

The activation of VRAC by intracellular GTPγS suggests that GTP is part of the activation pathway, possibly via small GTP-binding proteins [107]. This is consistent with the finding that Rho and Rho kinase (ROCK) are required for VRAC activation but cannot itself activate the channel (hence denoted permissive pathways) in several cell types [114–117]. Other

signaling pathways implicated in VRAC activation or modulation have been reviewed elsewhere [14, 22, 49, 56] and will only briefly be outlined here. They include bona fide signaling molecules such as phosphatidyl-inositol-3-kinase (PI3K) [118] and tyrosine kinases [107, 119–122], membrane lipids including cholesterol, various lipid-derived signaling molecules, the actin cytoskeleton [114, 123, 124], and other structural proteins including annexin-II [125] and caveolin-1 [126, 127].

VRAC physiology and pathophysiology

By far the most widely described function of VRAC is its essential role in RVD and hence cell volume homeostasis, in most cell types studied [14, 128, 129]. In addition, however, VRAC has been assigned a wide range of other important physiological functions. These include, but are not limited to, roles in electrogenesis, cell proliferation, angiogenesis, cell motility, and apoptosis (summarized in Fig. 2). These functions have been widely reviewed, and only a brief overview of pertinent aspects will be given below. It should be kept in mind that because of the hitherto elusive molecular identity of VRAC, conclusions on its function and dysfunction have drawn heavily on pharmacology, and it will be important to validate the proposed roles using specific molecular tools.

Roles of VRAC in electrogenesis

Studies in vascular endothelial cells have demonstrated that VRAC plays a central role in electrogenesis and hence is important for offsetting and regulating the driving forces for other ion channels and transporters [22]. While mainly studied in endothelial cells, obviously, VRAC inhibition will in general hyperpolarize cells with a depolarized membrane potential (V_m), and its activation will depolarize cells with a more negative membrane potential. As discussed in [22], the resting

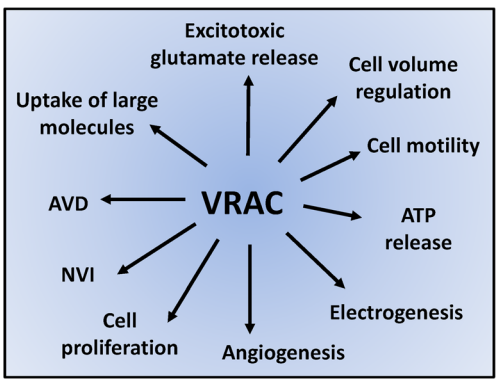

Fig. 2 Physiological and pathophysiological roles of VRAC. The figure illustrates the main proposed roles of VRAC in physiological and pathophysiological processes. See text for details. *AVD* apoptotic volume decrease, *NVI* necrotic volume increase

V_m of at least some endothelial cell types has been shown to exhibit a bimodal distribution, with one population with a resting V_m of −70 to −60 mV which is dominated by a K^+ conductance, and another in which the Cl^- conductance is dominating, and which consequently exhibits a resting V_m of −40 to −10 mV. In macrovascular bovine pulmonary aortic endothelial cells (BAEC), resting membrane currents are dominated by a combination of an inwardly rectifying K^+ (IRK) current and a VRAC Cl^- current (Fig. 3a–c), and inhibition of VRAC by mibefradil elicits hyperpolarization because the IRK current is now dominating (Fig. 3d; for details see [10, 22, 72]). Similarly, in calf pulmonary artery endothelial (CPAE) cells, mibefradil induces rapid hyperpolarization [99]. In general, however, the electrogenic effects of VRAC are understudied given their likely physiological importance and should be addressed in further detail and in a wider range of cell types.

Roles of VRAC in apoptotic cell death and chemotherapy resistance

A role for VRAC in apoptotic cell death has been demonstrated in multiple studies in a variety of cell types. Apoptosis following activation of the intrinsic apoptotic pathway (e.g., by staurosporine, STS) or of the extrinsic pathway [by death receptor ligands, e.g., Fas ligand (FasL) or TNFα (Fig. 4 upper scheme)] is causatively associated with persistent cell shrinkage [133]. This normotonic shrinkage has been denoted apoptotic volume decrease (AVD) [51, 134]. Thus, VRAC is activated by apoptotic stimuli, although intracellular ionic strength is increasing during apoptotic stress. One signal that can lead to VRAC activation under these conditions is ROS [53]. The AVD process is accompanied by efflux of KCl and osmotically obliged water [134] and involves VRAC activation [53, 135]. Additionally, pharmacological inhibition of VRAC inhibits apoptosis induced by STS [51, 136], FasL [51], TNFα [51], cisplatin [50], ROS [53, 137], and ischemia-reperfusion [138]. Conversely, isovolumetric cell shrinkage is sufficient to induce apoptosis [134, 139, 140]. It is therefore interesting to note that VRAC is downregulated in several drug-resistant cancer cell types, resulting in a decreased propensity for apoptosis [141–143]. Notably, in human ovarian cancer cells, cisplatin resistance was found to correlate with reduced swelling-activated taurine efflux and reduced expression of LRRC8A [143]. Recent findings from the Jentsch laboratory based on The Cancer Genome Atlas (TCGA) data suggested that while LRRC8A expression levels had no effect on survival of ovarian cancer patients treated with platinum drugs, reduced expression of LRRC8D correlated with reduced survival, consistent with the important role of this subunit in cellular cisplatin uptake [52, 61]. Collectively, the findings above show that VRAC is important for the cellular response to apoptotic stimuli. The recent

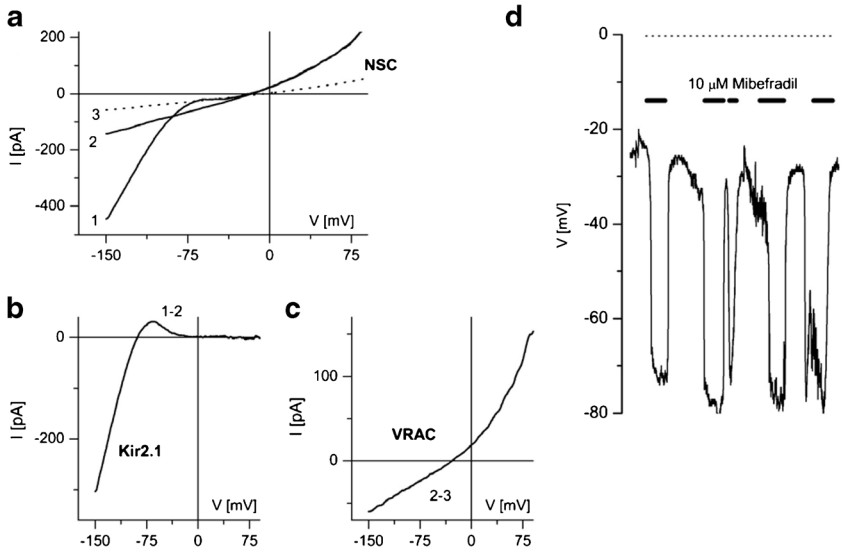

Fig. 3 Roles of VRAC in endothelial cell electrogenesis. The figure illustrates aspects of electrogenesis in non-stimulated bovine aortic pulmonary endothelial cells (BPAEC). **a** Current–voltage relationships from linear voltage ramps (from −150 to +100 mV), (*1*) under control conditions, (*2*) in the presence of 1 mM Ba^{2+} to inhibit the inwardly rectifying potassium (IRK) current, and after osmotic shrinkage (100 mM mannitol) to inhibit VRAC, still in the presence of Ba^{2+} (*3*). The remaining current is a nonselective cation (NSC) current. **b** The difference current between conditions *1* and *2* is IRK, mediated through Kir2.1 [130, 131]. **c** The VRAC current is the difference current between conditions *2* and *3*. **d** The resting V_m reflects the respective contributions of the three conductances. Upon inhibition of VRAC by Mibefradil (10 μM), the K^+ conductance becomes dominant and the membrane hyperpolarizes. The figure is modified from [22], with permission, and is based on data from [132] (panels **a-c**) and [72] (panel **d**)

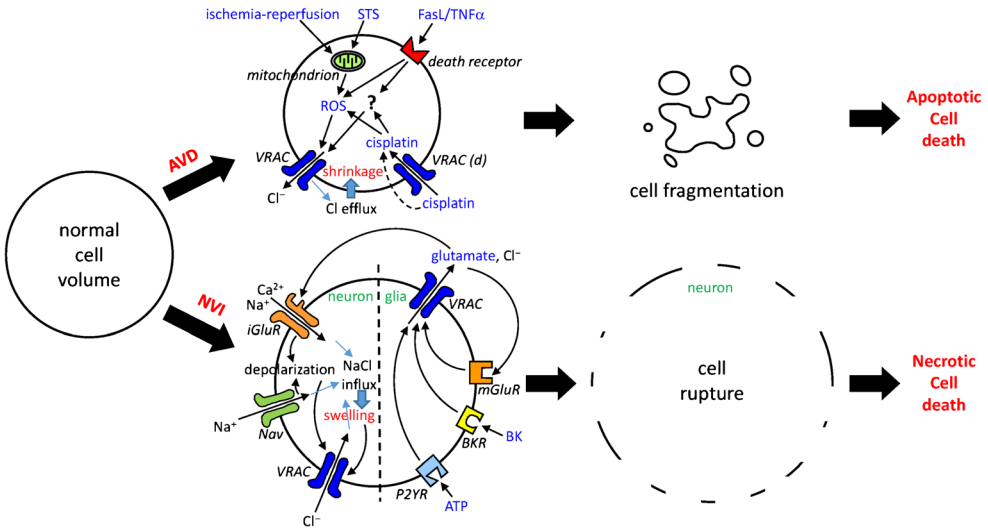

Fig. 4 Roles of VRAC in AVD and NVI. *Upper scheme:* roles of VRAC activity in the AVD in response to STS, FasL/TNFα, ischemia-reperfusion, and cisplatin. Under these conditions, VRAC is normotonically activated, resulting in a cell shrinkage that is necessary for the ensuing apoptotic cell death. In addition, VRAC serves as an uptake pathway for cisplatin. *Lower scheme:* roles of VRAC activity in NVI in response to glutamate-induced excitotoxicity and in glial glutamate release in response to glutamate-, BK-, and ATP-induced activation of mGluR, BKR, and P2YR, respectively. VRAC-mediated glutamate release further enhances, in a positive feedback manner, the release of glutamate via activation of mGluR in glial cells. Under these conditions, cell swelling induced by NaCl uptake further enhances neuronal VRAC activity but elicits Cl^- inflow rather than efflux due to the extensive depolarization resulting from iGluR activation. One of important characteristics of necrosis is the release of harmful or inflammatory factors to surrounding cells and tissues from the dying cells due to cell rupture. It must however be noted that numerous cell signaling mechanisms are involved in the processes between NVI, cell rupture and necrosis, as well as between AVD and cell fragmentation. *AVD* apoptotic volume decrease, *BK* bradykinin, *BKR* BK receptor, *FasL* Fas ligand, *iGluR* ionotropic glutamate receptor cation channel, *mGluR* metabotropic glutamate receptor, Na_v voltage-gated Na^+ channel, *NVI* necrotic volume increase, *P2YR* purinergic type 2Y receptor, *ROS* reactive oxygen species, *STS* staurosporine, *TNFα* tumor necrosis factor; *VRAC(d)* LRRC8 heteromer containing LRRC8D. The figure is modified from [49], with permission, and is based on data from [50–53] (upper scheme) and [54–58] (lower scheme)

demonstration that VRAC heteromers containing LRRC8D represent an important uptake mechanism for cisplatin and carboplatin shows that this at least in part reflects that VRAC is important for the cellular uptake of these compounds. On the other hand, the VRAC dependence of cell death in response to STS- or FasL treatment, ROS, or isotonic Cl^- efflux [51, 139, 144, 145] confirms the role of VRAC in the cell death process per se, as these stimuli are unlikely to be dependent on VRAC-mediated drug uptake (Fig. 4 upper scheme). While the broader relevance of VRAC in cancer clearly needs to be defined, it is interesting to note that a recent study of genes essential to net growth of human CML and Burkitt's lymphoma cell lines identified LRRC8A, C, D, and E as non-essential for this process ([146], Suppl. Tables 1–2). This is in congruence with the possibility that there may be other molecular candidates for VRAC than the LRRC8 family. However, it is equally consistent with the known roles of other types of Cl^- currents than VRAC (e.g., Ca^{2+}-activated Cl^- currents, see above) in RVD as well as in growth in some cell types and with the contributions of other transporters, e.g., KCl cotransporters, to these processes (see [14]).

Roles of VRAC in CNS function and necrotic cell death under excitotoxicity

As discussed in further detail by Mongin and coworkers elsewhere in this volume, VRAC is functionally expressed in brain neurons, astrocytes, and microglia (see [56]). In neurons, activation of voltage-gated Na^+ channels and ionotropic glutamate receptor cation channels (iGluR) leads to cell swelling, thereby activating VRAC (Fig. 4 lower scheme). Whereas the role of VRAC after cell swelling associated with physiological neuronal firing activity remains incompletely understood, its role in neuronal RVD has been widely studied (see [56]). As noted above, in addition to Cl^-, VRAC carries amino acids such as taurine, glutamate, and aspartate [92, 147–151]. This has important pathophysiological consequences because VRAC activation during, e.g., ischemic insults such as stroke causes swelling-induced neurotransmitter release from glia cells, contributing to excitotoxic damage [92, 148–154] (Fig. 4 lower scheme).

Necrotic cell death occurs in parallel with a marked normotonic cell swelling which has been denoted necrotic volume increase (NVI) [134, 155]. Neurotoxicity caused by prolonged exposure to excessive glutamate released from glial cells is termed excitotoxicity [156] and is associated with stroke, cerebral ischemia, brain trauma, and some neurodegenerative disorders, including epilepsy and Alzheimer's, Huntington's, and Parkinson's diseases [157]. Under excitotoxic conditions, neuronal swelling is induced by water inflow driven by Na^+ influx via iGluR and Cl^- influx via $GABA_A$ receptor anion channels, in turn, leading to activation of VRAC as described above (Fig. 4 lower scheme). Glutamate also activates mGluR, which enhance VRAC activity in a G-protein-dependent manner [56] (Fig. 4 lower scheme). In addition, ATP (acting as a neuro- and gliotransmitter) and BK (acting as an inflammatory mediator) also activate VRAC via G-protein-coupled receptors in a manner independent of cell swelling [54, 55, 58]. Once activated under these conditions, VRAC serves as a pathway not for volume-regulatory Cl^- efflux but for swelling-exacerbating Cl^- inflow because of the prominent V_m depolarization produced by iGluR activation (Fig. 4 lower scheme). This "reverse-mode" operation of VRAC leads to NVI and necrotic cell death in neurons [57].

VRAC in cell cycle progression and proliferation

VRAC currents are differentially regulated through the cell cycle, and inhibition of VRAC has been shown to inhibit proliferation in a wide range of cell types [111, 158–161]. Specifically, in SiHa human cervical cancer cells, VRAC inhibition resulted in G0/G1 arrest and delayed G1-S transition [160]. The precise cell cycle phase affected may differ between species: In nasopharyngeal carcinoma cells, VRAC activity was found to be downregulated in S phase compared to G1 and M [162]. In contrast, in SiHa cells, VRAC activity was reported to be increased in S compared to G0 and G1 [160], and a similar pattern was found in ELA cells [91]; for a review, see [163]. Also in congruence with a role of VRAC in control of proliferation status, downregulation of the VRAC current has been shown to be required for muscle cell differentiation [164]. While these studies need to be revisited at the molecular level given the unspecific nature of VRAC inhibitors, these data point to the importance of VRAC for cell cycle progression. The precise mechanism(s) remain to be elucidated, but several can be envisaged: The importance of V_m changes in proliferation have long been known, and the role of VRAC is paralleled by an important role of K^+ channels in cell proliferation [165]. Additionally, specific cell volume changes have been assigned important roles in cell cycle progression [166–170]. Interestingly, it was recently suggested that G1/S progression may involve a size-discriminatory process in G1, such that cells exit G1 with similar sizes [166].

VRAC in cell migration and angiogenesis

Another important physiological role of VRAC is the regulation of *cell migration*, which in several cell types is inhibited by VRAC inhibitors [171–174], presumably at least in part reflecting the involvement of local cell volume changes in cell motility (see [175]). Notably, a wide array of VRAC inhibitors attenuate the formation of new blood vessel in several model systems, e.g., matrigel-tube formation assay (in vitro), fibrin gel assay, and chorioallantoic membrane (CAM) assay (in ovo) [176, 177]. While inhibition of migration and/or

proliferation could contribute to the observed effects of VRAC in angiogenesis, this may render VRAC inhibitors interesting in the context of angiogenesis inhibition in, e.g., cancer.

Concluding remarks

This review has provided an overview of the current knowledge on VRAC biophysical properties, pharmacology, regulation, physiology, and pathophysiology–the great majority obtained before the recent breakthrough in the understanding of the molecular identity of VRAC. These studies can now be used as an important starting point for novel discoveries and structure-function understanding, based on the identification of LRRC8A as an essential component of VRAC. Centrally open questions include the precise identity of the VRAC pore; whether other molecular entities than the LRRC8 proteins contribute to VRAC, perhaps via interactions with the LRRC8 proteins; the mechanisms through which VRAC is activated by cell volume perturbations and other stimuli; and, of course, the molecular validation of the multiple roles of VRAC regulation and dysregulation in human physiology and pathophysiology that have so far relied on pharmacological tools. The coming years will surely see a surge of new replace "important" with "pivotal" (to avoid two times "important" in the same sentence) discoveries about the functions of this ubiquitously important channel.

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