A SWELL time to develop the molecular pharmacology of the volume-regulated anion channel (VRAC)

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\textbf{ABSTRACT}

Newly emerging roles of LRRC8 volume-regulated anion channels (VRAC) raise important questions about the therapeutic potential of VRAC in the treatment of epilepsy, type 2 diabetes, and other human diseases. A critical barrier to evaluating whether VRAC represents a viable drug target is the lack of potent and specific small-molecule inhibitors and activators of the channel. Here we review recent progress in developing the molecular pharmacology of VRAC made by screening a library of FDA-approved drugs for novel channel modulators. We discuss the discovery and characterization of cysteinyl leukotriene receptor antagonists Pranlukast and Zafirlukast as novel VRAC inhibitors, and zinc pyrithione (ZPT), which apparently activates VRAC through a reactive oxygen species (ROS)-dependent mechanism. These ongoing efforts set the stage for developing a pharmacological toolkit for probing the integrative physiology, molecular pharmacology, and therapeutic potential of VRAC.

\textbf{Overview of the volume-regulated anion channel (VRAC)}

A cell’s ability to regulate its volume in response to osmotic stress arose early in evolution and allowed complex organisms to escape the primordial ocean and invade the land [1–5]. In vertebrate cells, osmotic cell swelling activates a complex process known as regulatory volume decrease, or RVD, where ion channels and transporters mediate K\textsuperscript{+}, Cl\textsuperscript{−}, organic osmolyte, and water efflux that returns cell volume back to normal [6]. A key player in RVD is the volume-regulated anion channel, or VRAC [7]. VRAC is also known as the volume-sensitive organic osmolyte-anion channel (VSOAC) owing to its role in transporting osmolytes such as taurine glutamate. However, we will use the more common name, VRAC, throughout this review.

After more than 30 years of studying VRAC biophysical, regulatory, and pharmacological properties in diverse cell types, the genes encoding the channel were discovered by two independent laboratories using similar genome-wide, siRNA knockdown, high-throughput screening platforms (discussed below) \cite{8,9}. The cell-based screening assay utilizes a halide-sensitive yellow-fluorescent protein (YFP) variant that undergoes fluorescence quenching as iodide moves through VRAC and into the cytoplasm where YFP is expressed. Knock-down of the leucine-rich repeat containing 8A (LRRC8A) gene reduced YFP quenching by iodide, indicating that LRRC8A, also known as SWELL1 \cite{8,9}, is an essential subunit of the VRAC complex. Other members of the gene family are LRRC8B, LRRC8C, LRRC8D, and LRRC8E, none of which give rise to channel activity when expressed alone; however, when co-expressed with LRRC8A, LRRC8C, LRRC8D, or LRRC8E gives rise to swelling-activated anion channels with distinctive functional properties \cite{8,10}. Cryo-electron microscopy evidence indicates that LRRC8A and LRRC8D form homohexamers when expressed alone in heterologous expression systems lacking other LRRC8 subunits \cite{9,11–15}.

The ability to silence the function of all VRAC isoforms by deleting LRRC8A provides a convenient way to generate knockout mice and cell lines lacking VRAC activity. This approach has been used widely...
to generate new insights into the role of VRAC in immune cell function, astrocyte physiology, sperm development, cell signaling in adipocytes, glucose sensing and insulin release by pancreatic beta cells, vascular smooth muscle cell signaling, and cancer drug transport [16–18]. The reader is referred to several recent review articles covering these topics in greater detail [7,19–21]. In some cases, these new discoveries have implicated VRAC as novel therapeutic targets for disease. For example, knockout of LRRC8A specifically in astrocytes protects from brain injury following ischemic stroke, likely due to the channel’s role in glutamate release and excitotoxic neuronal cell death. This suggests VRAC might represent a novel drug target for stroke and other diseases associated with neuronal excitotoxicity. In pancreatic beta cells, VRAC enhances insulin secretion following glucose uptake suggesting the channel could be a novel drug target for type 2 diabetes. In support of this idea, Sah and colleagues recently reported that a small molecule that enhances the stability of VRAC in the cell membrane promotes glucose responsiveness and insulin release from beta cells [22]. Whether or not a frank activator of VRAC would have similar effects is not yet known.

Critically evaluating the therapeutic potential and safety of targeting VRAC for treating stroke, diabetes, and other diseases will require potent and selective channel modulators with suitable metabolic and pharmacokinetic profiles to enable channel modulation in vivo. However, the current molecular pharmacology of VRAC falls short of this requirement, consisting mostly of weak and nonspecific chloride channel blockers that have myriad off-target activities. Below, we provide a brief overview of the current pharmacology of VRAC and then discuss ongoing efforts to develop a new generation of channel modulators.

**Most VRAC inhibitors are neither potent nor specific**

Many of the inhibitors that have been used to study VRAC function were discovered serendipitously while searching for blockers of other anion transport pathways [23–27]. Classical anion transporter/channel inhibitors such as SITS (4-acetamido-4’-isothio-cyanato-2,2’-stilbenedisulfonic acid), DIDS (4,4’-diisothiocynano-2,2’-stilbenedisulfonic acid), NFA (niflumic acid), FFA (fluflemic acid), NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid), DPC (diphenylamine-2-carboxylate), 9-AC (9-Anthracencarboxylic acid), NPA (N-phenylanthracilic acid), inhibit VRAC with IC₅₀s in the single to 100s of micromolar range [28–34], but have off-target effects on numerous other transport pathways.

The most potent and specific inhibitor of VRAC known to date is the ethacrynic-acid derivative DCPIB (4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl)oxybutyric acid) [35] (Figure 1). DCPIB has been shown to inhibit VRAC in numerous cell types, including HEK293 cells, HELA cells, HCT116 cells, calf bovine pulmonary artery endothelial cells, mouse astrocytes, Xenopus oocytes, Guinea-pig atrial cardiomyocytes, and rat pancreatic beta cells, in a voltage-independent, fully reversible manner with an IC₅₀ in the range of 2–5 μM [31,35–41]. DCPIB is preferred over other VRAC inhibitors due to its specificity toward VRAC over other Cl⁻ channels such as CFTR, CaCCs, CLCs, Maxi-Cl, and PAC [33,35,42,43]. However, it has been shown to have several off-target effects inward rectifier K⁺ channels, tandem-pore K⁺ channels, H⁺/K⁺-ATPase, connexin hemichannels, glutamate transporter GLT-1, and inhibits mitochondrial respiration [44–48].

The K⁺Cl⁻ cotransporter antagonist, R-(+)-(2-n-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1 H-inden-5-yl)-oxy] acetic acid (DIOA) is structurally related to DCPIB, only differing in the length of its oxy-carbonic acid group (DCPIB has an extra carbon in the linker). DIOA inhibits VRAC in rat thymocytes [49] and HeLa cells with an IC₅₀ of 20 mM [33]. The differences in potency between DCPIB and DIOA indicate the length of the oxy-carbonic acid group is likely important for VRAC inhibition.

There are several other compounds that are known to inhibit VRAC but have been used less frequently. For example, the acidic di-aryl urea compound NS3728 inhibits VRAC with an IC₅₀ of 400 nM but has off-target effects on calcium-activated Cl⁻ channels [30,50]. The natural phenol, phloretin inhibits VRAC in a voltage-independent and reversible manner with an IC₅₀ of 30 μM [51]. Inhibition is only observed when phloretin was
applied extracellularly; intracellular application through the pipette solution did not inhibit VRAC [51]. Phloretin also inhibits CFTR, various cation channels, aquaporins, and transporters [51]. The estrogen receptor modulator, tamoxifen, has been shown to inhibit VRAC in astrocytes, endothelial cells, epithelial cells, macrophages, fibroblasts, mouse cortical collecting duct cells with IC_{50} in the low single-micromolar range [29,30,52–57], but interestingly does not inhibit VRAC in neurons [58–60]. Finally, the gap junction/hemichannel inhibitor, carbenoxolone (CBX), inhibits VRAC with an IC_{50} of 15 μM [61], potentially through interactions with the extracellular side of the channel [62]. Unfortunately, none of these VRAC inhibitors are potent or selective enough for evaluating the druggability and therapeutic potential of VRAC for treating diseases.

**A high-throughput screening assay for discovering novel VRAC modulators**

A major technological hurdle to discovering new chemical probes of VRAC is the low throughput of manual patch clamp electrophysiology, which is too slow and labor intensive to enable large compound library screening. And although high-throughput electrophysiology instruments exist and are widely used in the pharmaceutical industry, this technology is generally prohibitively expensive for library screening in academic laboratories. The Verkman laboratory developed a series of genetically encoded, yellow-fluorescent protein (YFP)-based anion sensors that effectively overcame these barriers. They developed a double mutant (YFP-H148Q/I152L) that exhibits bright, stable fluorescence that is rapidly quenched when iodide is transported into the cell by a chloride channel or transporter of interest [63,64]. David Weaver and colleagues further optimized tempe rate stability and fluorescence properties of YFP by introducing a third mutation (YFP-F46L/H148Q/I152L) [65]. YFP-quenching assays have been used extensively the develop the pharmacology of the GABA_{A} receptor, CFTR, calcium-activated chloride channels [66], and was key in discovering the genes that encode VRAC [8,9].

Figure 2(a) illustrates how the YFP-quenching assay can used to report endogenously expressed VRAC activity in a small-molecule library screen. Cells stably expressing YFP-F46L/H148Q/I152L are plated in black-walled, clear-bottomed, 384-well assay plates and cultured overnight to establish an adherent cell monolayer. The following day, the cell culture media is removed from the plate and exchanged for isotonic buffered saline. The cells are subsequently treated with hypotonic buffer containing compounds or solvent control (e.g. DMSO) to induce cell swelling and VRAC activation, and then treated with iodide-
containing buffer to induce VRAC-dependent iodide influx and YFP quenching. Small molecules that inhibit or activate VRAC should reduce or enhance the rate and extent of iodide-induced quenching, respectively.

**Discovery of Pranlukast and Zafirlukast as novel VRAC inhibitors**

We employed the YFP-F46L/H148Q/I152L-quenching assay to screen a library of 1,184 FDA-approved drugs for novel modulators of native VRAC expressed endogenously in HEK-293 cells. We selected this library for a pilot screen for several reasons, including its relatively small size, the potential of identifying compounds with drug metabolism and pharmacokinetic properties that are suitable for in vivo experiments, and possibility of identifying drugs that could be repurposed for exploring the therapeutic potential of VRAC in preclinical models.

The most potent inhibitor identified in the FDA library screen was Pranlukast (Figure 1), a cysteinyl leukotriene 1 (CysLT1) receptor antagonist used to inhibit bronchospasms in the treatment of asthma [67,68]. Pranlukast is highly selective for CysLT1 over CysLT2 receptors with IC$_{50}$ values of 23 nM and 3,620 nM, respectively [69,70]. In whole-cell patch clamp experiments on HEK-293 cells, Pranlukast inhibited swelling-activated VRAC currents dose-dependently with an IC$_{50}$ of ~3 μM and a maximal inhibitory efficacy of 50% at 10 μM. Inhibition was voltage-independent, occurred with a time constant of ~60 sec, was fully reversible, and was associated with changes in current inactivation kinetics at +120 mV. Interestingly, the structurally distinct CysLT1 receptor antagonist, Zafirlukast (Figure 1), also inhibited VRAC, albeit with an IC$_{50}$ of ~17 μM and nearly 100% efficacy at 100 μM.

The discovery that two structurally distinct CysLT1 receptor antagonists inhibit VRAC activity raised the possibility that VRAC inhibition occurred indirectly through modulation of leukotriene signaling in HEK-293 cells. In support of this idea, Holm and colleagues had previously shown that 60 μM Zafirlukast inhibits RVD and release of the organic osmolyte, taurine, following hypotonic cell swelling of A549 lung epithelial cells [71]. Taken together with their additional observation that an inhibitor of 5-lipoxygenase, the major source of inflammatory leukotrienes that cause asthma, also inhibits RVD and taurine release, led the authors to propose a model in which osmotic cell swelling stimulates the release of leukotrienes which, in turn, signal through CysLT1 to promote VRAC activation and RVD. According to their model, antagonists of CysLT1 should inhibit swelling-
induced VRAC activation, as observed in our study [72]. We therefore set out to determine if Pranlukast-dependent inhibition of VRAC in HEK-293 cells is dependent on leukotriene signaling.

CysLT1 is a Gq-coupled GPCR that leads to intracellular calcium release following receptor stimulation, phospholipase C activation, and liberation of IP3 from membrane PIP2. Thus, if Pranlukast inhibits VRAC indirectly through antagonism of leukotriene signaling, the molecular components of this pathway should be functionally expressed in HEK-293 cells. However, we found that CYSLTR1 mRNA is not detectably expressed in HEK-293 cells, confirming expression data published on The Human Protein Atlas database (www.proteinatlas.org). Additionally, although acetylcholine was able to induce intracellular calcium release through stimulation of Gq-coupled M3 muscarinic receptors endogenously expressed in HEK-293 cells, the high-affinity CysLT1 agonist, LTD4, neither induced calcium release nor promoted VRAC activation under isotonic conditions. Taken together, these data indicate that the CysLT1 signaling pathway is not functionally expressed in HEK-293 cells and that the inhibitory effects of Pranlukast and Zafirlukast on VRAC occur independently of leukotriene signaling.

**Discovery of zinc pyrithione (ZPT) as a positive modulator of VRAC activity**

The anti-dandruff, anti-fouling agent zinc pyrithione (ZPT) (Figure 1) was found in the FDA library screen to dose-dependently increase the rate and extent of YFP (F46L/H148Q/I152L) quenching (Figure 2(b,c)) with an EC50 of 5.7 μM, suggesting the compound acts as a positive modulator of VRAC currents expressed in HEK-293 cells. This was confirmed in whole-cell patch clamp experiments, which demonstrated that ZPT not only potentiated the rate of cell swelling-induced VRAC currents in HEK-293 cells and HCT116 cells but also activated VRAC currents that have reached steady state after swelling and in the absence of swelling.

Given that ZPT is the first-known small-molecule activator of VRAC, understanding its mechanism of action could provide important insights into channel structure-function relationships and regulatory mechanisms. When dissolved in an aqueous buffer, ZPT can dissociate into Zn2+ and pyrithione or remain as a ZPT complex, prompting us to examine the effects of each of these components on VRAC activity. Bath application of free Zn2+ or free pyrithione failed to activate VRAC in patch clamp experiments, whereas pre-mixing the two components before cell treatment led to channel activation in the absence of hypotonic cell swelling. This observation supports a model in which the Zn2+-pyrithione complex mediates the channel activatory response to ZPT.

The ZPT complex could activate VRAC through at least two distinct molecular mechanisms, namely, 1) direct ligand interactions with the channel protein or 2) modulation of cell signaling pathways that regulate VRAC. Although there is currently no experimental evidence to support a direct mechanism of action, the ZPT complex is known to potentiate the activity KCNQ7 (Kv7.2) potassium channels, potentially through ligand interactions with S5 and S6 transmembrane domains. To address an indirect mechanism, we explored wither the effects of ZPT on VRAC are mediated through a reactive oxygen species (ROS)-dependent processes since ZPT induces ROS production in some cell types [73,74] and ROS is known to activate VRAC currents [75–77]. Pre-treatment of cells with the ROS scavenger N-acetylcysteine (NAC) or NAD(P)H inhibitor diphenylene-iiodonium (DPI) dramatically reduced the effects of ZPT on VRAC, suggesting the ZPT complex activates the channel indirectly via ROS. There is an emerging literature that further supports such a mechanism. For example, LRRC8A co-localizes and co-immunoprecipitates with the NAD(P)H subunits Nox2, Nox4, and p22phox. ROS induced with chloramine-T activates LRRC8A/C channels through oxidation of intracellular cysteine residues but inhibits LRRC8A/D channels through complex mechanisms that are not yet resolved [78]. It will be important to determine if the effects of ZPT are LRRC8-subunit and cell-type dependent and identify amino acid residues that mediate ZPT’s effects on VRAC activity. Furthermore, considering that ZPT is known to induce apoptotic cell death in some cell types
[73,74,79,80], and that VRAC plays a key role in apoptotic cell shrinkage [81], future studies should determine if VRAC is part of ZPT’s therapeutic mechanism of action.

**Use of novel LRRC8 chimeras for probing small-molecule mechanism of action**

Whether Pranlukast, Zafirlukast, and ZPT modulate VRAC activity through direct interactions with the channel protein or indirectly via modulation of regulatory-signaling pathways is currently unknown. A commonly used approach to study the molecular pharmacology of an ion channel involves characterizing how engineered mutations alter the pharmacology of the channel in a heterologous expression system. However, VRAC presents at least two unique challenges for studying structure–pharmacology relationships using this approach. First, the LRRC8 gene family is ubiquitously expresses in vertebrate cells, including commonly used expression systems such as immortalized cells lines and *Xenopus laevis* oocytes, thus, the “background” expression of endogenous wild-type subunits could potentially mask the effects of engineered mutations on heterologously expressed subunits. This limitation can be circumvented with the judicious use of cell lines in which individual or all 5 LRRC8 genes have been knocked out using CRISPR-mediated technology [8]. A second challenge is that the stoichiometry and molecular arrangement of subunits in a native channel is unknown and possibly variable among cell types [7]. It is conceivable and even likely that the effect of an engineered mutation on channel pharmacology will be influenced by the number and position of mutated subunits present in a channel complex. One solution is to study the pharmacology in homomeric channels comprised one type of subunit. Kern et al. used single-particle cryo-electron microscopy to solve the co-structure of LRRC8A and the best-in-class VRAC inhibitor DCPIB [12]. They observed that DCPIB binds to arginine 103 (R103) of LRRC8A to block the narrowest part of the extracellular pore mouth via a “cork in a bottle” mechanism.

Despite several cryo-EM structures of LRRC8A homohexameric channels being published [11–15], the functional, regulatory, and pharmacological properties of LRRC8A homomeric channels were only recently reported [41] due to the absence of channel activity under normal recording conditions. Unlike native VRAC currents, LRRC8A channels are poorly activated by low intracellular ionic strength and are insensitive to osmotic cell swelling under normal intracellular ionic strength conditions. However, combining low intracellular ionic strength and cell swelling gives rise to modest channel activity, enabling characterization of
LRRC8A pore properties. One of the most surprising characteristics of LRRC8A homomeric channels is its weak and voltage-dependent block by DCPIB, which is in striking contrast to the strong and voltage-independent block of native VRAC currents. As predicted from the LRRC8A-DCPIB co-structure [12], mutation of R103 to phenylalanine (R103F) abolished DCPIB-dependent inhibition of LRRC8A but had no effect on DCPIB block of LRRC8A (R103F)/LRRC8C heteromeric channels. Furthermore, a chimeric channel comprising mostly LRRC8C and a short 25-amino acid stretch of intracellular loop 1 (Figure 3) from LRRC8A (LRRC8C-8A (IL125)) that lacks an arginine at the equivalent position of LRRC8A(R103) is strongly inhibited by DCPIB. These observations raise concerns about the utility of LRRC8A homomeric channel structures in guiding mutagenesis-based analysis of structure-function-pharmacology relationships.

The use of LRRC8C-8A (IL125) or other chimeras provides several important advantages over LRRC8A for structure–function studies of VRAC regulation and pharmacology. First, LRRC8C-8A (IL125) channels exhibit native-like activation by low intracellular ionic strength and cell swelling, and inactivation by hypertonic cell shrinkage. Second, their DCPIB sensitivity closely resembles that of native VRAC currents. Third, and importantly, they form homomeric channels that circumvent complicating issues with subunit stoichiometry and position described above. Solving the structure of LRRC8C-8A (IL125) could provide a more “physiological” model for understanding how VRAC channels are regulated by cell volume changes and modulated pharmacologically.

Conclusions

The discovery of the LRRC8 gene family that encodes VRAC has generated renewed interest in understanding the molecular physiology and therapeutic potential of these channels in human diseases. Despite proving useful in the initial characterizations of VRAC physiology, its pharmacology remains underdeveloped. DCPIB was initially described as a VRAC inhibitor 20 years ago and is still the best-in-class inhibitor to date, even with its myriad of off-targets. Our lab has only begun to better understand how native VRAC may be modulated by small-molecule compounds utilizing the robust YFP-quenching assays previously established as a functional readout of chloride channel activity [72,82]. Others have used cryo-EM to identify putative binding sites of small-molecule VRAC inhibitors and using the gained structural insight to develop even more potent channel blockers [12,22]. Protein engineering approaches have generated LRRC8 chimeras that may serve as a complementary approach to identify putative binding sites of known VRAC modulators [83]. Furthermore, these chimeras could be used to study the pharmacological modulation of individual LRRC8 subunits. Given the emerging genetic knockout evidence that certain VRAC subunits subtypes (i.e. LRRC8C-E) play specific physiological roles, subunit-specific modulators would be useful in further testing these hypotheses. While VRAC’s pharmacology is still in its infancy, ongoing studies are paving the way to generate tools to study these important channels and ultimately test if targeting VRAC is therapeutic for human disease.

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