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Cell Volume-Activated and Volume-Correlated Anion Channels in Mammalian Cells: Their Biophysical, Molecular, and Pharmacological Properties

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Abstract.....	50
I. Introduction.....	50
A. Classification and Terminology of Anion Channels.....	50
B. Primary Actions of Anion Channel Opening.....	51
C. Volume-Controlling Action of Anion Channels.....	52
II. Volume-Activated Anion Channels.....	52
A. ClC-2-Type Chloride Channel.....	53
B. Volume-Sensitive Outwardly Rectifying Anion Channel.....	53
1. Phenotypical Properties.....	54
2. Activation Mechanisms.....	54
3. Molecular Identity.....	59
C. Maxi-Anion Channel.....	62
1. Phenotypical Properties.....	63
2. Activation Mechanisms.....	64
3. Molecular Identity.....	64
III. Volume-Correlated Anion Channels.....	66
A. Cystic Fibrosis Transmembrane Conductance Regulator.....	66
B. Calcium-Activated Chloride Channel.....	67
C. Acid-Sensitive Outwardly Rectifying Anion Channel.....	68
IV. Pharmacological Properties of and Distinctions between Volume-Activated Anion Channels and Volume-Correlated Anion Channels.....	70
A. ClC-2-Type Chloride Channel Blockers.....	70
B. Volume-Sensitive Outwardly Rectifying Anion Channel Blockers.....	71
C. Maxi-Anion Channel Blockers.....	74
D. Cystic Fibrosis Transmembrane Conductance Regulator Blockers.....	75
E. Calcium-Activated Chloride Channel Blockers.....	77
F. Acid-Sensitive Outwardly Rectifying Anion Channel Blockers.....	78
G. Their Pharmacological Distinctions.....	79

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V. Conclusions and Future Directions.....	79
Acknowledgments	79
References	79

Abstract—There are a number of mammalian anion channel types associated with cell volume changes. These channel types are classified into two groups: volume-activated anion channels (VAACs) and volume-correlated anion channels (VCACs). VAACs can be directly activated by cell swelling and include the volume-sensitive outwardly rectifying anion channel (VSOR), which is also called the volume-regulated anion channel; the maxi-anion channel (MAC or Maxi-Cl); and the voltage-gated anion channel, chloride channel (ClC)-2. VCACs can be facultatively implicated in, although not directly activated by, cell volume changes and include the cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) anion channel, the Ca²⁺-activated Cl[−] channel (CaCC), and the acid-sensitive (or acid-stimulated) outwardly rectifying anion channel. This article describes the phenotypical

properties and activation mechanisms of both groups of anion channels, including accumulating pieces of information on the basis of recent molecular understanding. To that end, this review also highlights the molecular identities of both anion channel groups; in addition to the molecular identities of ClC-2 and CFTR, those of CaCC, VSOR, and Maxi-Cl were recently identified by applying genome-wide approaches. In the last section of this review, the most up-to-date information on the pharmacological properties of both anion channel groups, especially their half-maximal inhibitory concentrations (IC₅₀ values) and voltage-dependent blocking, is summarized particularly from the standpoint of pharmacological distinctions among them. Future physiologic and pharmacological studies are definitely warranted for therapeutic targeting of dysfunction of VAACs and VCACs.

I. Introduction

Chloride is the most abundant anion in the living human body, constituting approximately 70% and 65% of total anionic species of the interstitial fluid and blood plasma, respectively. Although the intracellular solution consists of a large variety of other anionic species including organic anions, inorganic anion Cl[−] is the most predominant membrane-permeable anion. Therefore, the membrane-spanning channels for anionic electrolytes have often been called chloride channels. A number of chloride channel types have also recently been shown to participate in cell-to-cell signaling by serving as pathways for the release of intracellular

organic anions such as glutamate and ATP. Thus, the term *anion channel* (in lieu of *chloride channel*) is mainly used throughout this review.

A. Classification and Terminology of Anion Channels

Mammalian anion channels are ordinarily classified into six major groups according to their activation mechanisms (as listed in Table 1), although some minor types of anion channels may exist depending on the cell type. For example, the outwardly rectifying (depolarization-induced) Cl[−] channel in epithelial cells is often associated with unphysiological situations (for review, see Guggino, 1993) and prostaglandin E₂ (PGE₂)/nitric oxide/cGMP-activated small-conductance anion channels in gastric

ABBREVIATIONS: 9-AC, anthracene-9-carboxylic acid; A23187, 5-(methylamino)-2-((2*R*,3*R*,6*S*,8*S*,9*R*,11*R*)-3,9,11-trimethyl-8-[(1*S*)-1-methyl-2-oxo-2-(1*H*-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl)methyl)-1,3-benzoxazole-4-carboxylic acid; ABC, ATP-binding cassette; ANO, anoctamin; ASOR, acid-sensitive outwardly rectifying anion channel; [ATP]_i, intracellular free ATP concentration; AVD, apoptotic volume decrease; BSP, bromosulphophthalein; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; CaCC, Ca²⁺-activated Cl[−] channel; CaCC_{inh}-A01, 6-(1,1-dimethylethyl)-2-[(2-furanylcarbonyl)amino]-4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylic acid; CBX, carbenoxolone; CFTR, cystic fibrosis transmembrane conductance regulator; CFTR_{inh}-172, 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone; CHO, Chinese hamster ovary; [Cl[−]]_i, intracellular Cl[−] concentration; DASU-02, N-(4-methylphenylsulfonyl)-N'-(4-trifluoromethylphenyl)urea; DCPIB, 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid; DIDS, 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid; DIOA, *R*-(+)-[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]acetic acid; DPC, diphenylamine-2-carboxylate; DUOX, dual oxidase; *E*_{Cl}, equilibrium potential for Cl[−]; ER, endoplasmic reticulum; FFA, flufenamic acid; FRT, Fischer rat thyroid; GABA_AR, GABA_A receptor; GAP27, gap junction peptide 27; GlyH-101, *N*-(2-naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene)glycine hydrazide; GlyR, glycine receptor; GSH, glutathione; GTPγS, guanosine 5'-3-*O*-(thio)triphosphate; H7, 5-(2-methylpiperazin-1-yl)sulfonylisoquinoline dihydrochloride; HEK293, human embryonic kidney 293; HEK293T, human embryonic kidney 293 T; *I*-*V*, current-voltage; KCC, K⁺-Cl[−] cotransporter; LRR, leucine-rich repeat; Maxi-Cl, maxi-anion channel (also MAC); [Mg²⁺]_i, intracellular free Mg²⁺ concentration; NBD, nucleotide binding domain; NFA, niflumic acid; NKCC, Na⁺-K⁺-2Cl[−] cotransporter; NOX, NAD(P)H oxidase; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; NS3728, *N*-[3,5-bis(trifluoromethyl)-phenyl]-*N*-[4-bromo-2-(1*H*-tetrazol-5-yl)-phenyl] urea; NVI, necrotic volume increase; PGE₂, prostaglandin E₂; PGT, prostaglandin transporter; PKA, protein kinase A; PPQ-102, 6,7-dihydro-7,9-dimethyl-6-(5-methyl-2-furanyl)-11-phenylpyrimido[4',5':3,4]pyrrolo[1,2-*a*]quinoxaline-8,10(5*H*,9)-dione; PTK, protein tyrosine kinase; RD, regulatory domain; ROS, reactive oxygen species; RPE, retinal pigment epithelium; RVD, regulatory volume decrease; siRNA, small interfering RNA; SITS, 4-acetamido-4'-isothiocyano-2,2'-disulfonic stilbene; T16A_{inh}-A01, 2-[(5-ethyl-1,6-dihydro-4-methyl-6-oxo-2-pyrimidinyl)thio]-*N*-[4-(4-methoxyphenyl)-2-thiazolyl]acetamide; VAAC, volume-activated anion channel; VCAC, volume-correlated anion channel; VRAC, volume-regulated anion channel; VSOAC, volume-sensitive organic osmolyte anion channel; VSOR, volume-sensitive outwardly rectifying anion channel; WW781, 4-[4-[(1*E*,3*E*)-5-(1,3-dibutyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)penta-1,3-dienyl]-3-methyl-5-oxo-4*H*-pyrazol-1-yl]-benzenesulfonic acid.

TABLE 1
Mammalian anion channels: Classification and relation to cell volume and organic anion transport

Species	Channel Name	Abbreviation	Molecule
Ligand gated	Glycine receptor GABA _A receptor GABA _C receptor	GlyR GABA _A R GABA _C R	GlyR GABA _A GABA _C
Voltage gated	ClC-type chloride channel	ClC-1 ClC-2 (one of VAAC and VRAC) ClC-K1 ClC-K2	CLCN1 CLCN2 CLCNK1 CLCNK2
cAMP activated	Cystic fibrosis transmembrane conductance regulator	CFTR (one of VCAC and VSOAC)	CFTR
Ca ²⁺ activated		CaCC (one of VCAC)	TMEM16A/ANO1 TMEM16B/ANO2 TMEM16F/ANO6
Volume activated	Volume-sensitive outwardly rectifying anion channel Maxi-anion channel	VSOR (one of VAAC, VRAC, ^a and VSOAC) Maxi-Cl (one of VAAC, VRAC, ^a VSOAC, and MAC-1 ^b)	LRRC8A+8C/8D/8E(+ ^{7c}) SLCO2A1(+ ^{7c})
Acid activated	Acid-sensitive outwardly rectifying anion channel	ASOR (one of VCAC)	?

^aThe VRAC abbreviation used in this work (for volume-regulated anion channel) is sometimes also referred to as the volume-regulatory anion channel.

^bMaxi-Cl is the major type of maxi-anion channel (MAC-1) (see the text for details).

^cThe question mark represents some missing molecular component which may exist to reproduce a full set of phenotypes (see the text for details).

parietal cells (Sakai et al., 1989, 1995). Ligand-gated anion channels are called ionotropic receptors, because they are coupled to the glycine receptor (GlyR), GABA_A receptor (GABA_AR), or GABA_C receptor. Voltage-gated anion channels are formed by several types of ClC family members, including ClC-1, ClC-2, ClC-K1(Ka), and ClC-K2(Kb). Anion channels activated by intracellular signals cAMP and Ca²⁺ are called the cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel and the Ca²⁺-activated Cl⁻ channel (CaCC), respectively. Although CFTR activation is regulated by cAMP-induced activation of protein kinase A (PKA), its opening and closure are rather directly gated by binding of intracellular ATP and release of its hydrolytic product, respectively. Thus, CFTR may also be classified as a ligand-gated anion channel (Kirk and Wang, 2011). A distinct type of anion channel is the acid-sensitive outwardly rectifying anion channel (ASOR), which is quickly activated by extracellular acidification and characterized by sharp outward rectification. The remaining two types of anion channels are slowly activated by cell swelling and are referred to as the volume-sensitive anion channels: the volume-sensitive outwardly rectifying anion channel (VSOR) is distinctly characterized by mild outward rectification, whereas the most predominant type of maxi-anion channel (MAC; also called the Maxi-Cl, see section II.C), is distinctly characterized by a large unitary ohmic [current-voltage (*I*-*V*) linear] conductance of hundreds of picosiemens.

Among these mammalian anion channels, a number are implicated in cell volume changes. VSOR, Maxi-Cl, and ClC-2 are directly activated by cell swelling (Strange et al., 1996) and are thus collectively dubbed the volume-activated anion channels (VAACs) (Okada et al., 2009a,b) (Table 1). Although CFTR, CaCC, and ASOR cannot be directly activated by cell swelling per

se, they can be facultatively implicated in changes in cell volume. Here, these anion channels are collectively called the volume-correlated anion channels (VCACs) (Table 1). Although VSOR alone has often been called the volume-regulated anion channel (VRAC) (Table 1) or *I*_{Cl,swell}, this terminology may be misleading because Maxi-Cl and ClC-2 are also literally regulated by volume and activated by cell swelling. In addition, the VRAC abbreviation could also be confusingly assigned to volume-regulatory anion channels (Table 1). VRAC has also sometimes been referred to as the volume-sensitive organic osmolyte anion channel (VSOAC) (Table 1), taking its capability to release intracellular organic anions into consideration (Strange et al., 1996). However, VSOR, Maxi-Cl, and CFTR can serve as pathways for the efflux of organic signaling solutes such as glutamate and glutathione (GSH) (Hudson, 2001; Sabirov et al., 2016); therefore, they could also be called VSOACs (Table 1).

B. Primary Actions of Anion Channel Opening

The opening of anion channels on the plasma membrane results in the shift of membrane potential to the equilibrium potential for Cl⁻ (*E*_{Cl}) and the net transport of anions across the cell membrane. The former action is not necessarily coupled to the latter action for Cl⁻, because no electrochemical driving force is given for the Cl⁻ transport when the membrane potential happens to reach the *E*_{Cl} value.

The intracellular Cl⁻ concentration ([Cl⁻]_i) is normally controlled by the balance of counteracting actions of two types of electroneutral cation-chloride cotransporters (Fig. 1): the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC) and the K⁺-Cl⁻ cotransporter (KCC). The predominant activities of KCC and NKCC maintain the [Cl⁻]_i level below and above the *E*_{Cl} value, respectively (Russell, 2000; Blaesse et al., 2009; Payne, 2009). Since the [Cl⁻]_i is lower than the value determined by its

Fig. 1. Primary actions of opening of anion channels in either a KCC-dominated case (A) or an NKCC-dominated case (B) (see the text for details).

electrochemical equilibrium in mature central neurons and skeletal muscle cells, in which KCC is constitutively operating, opening of neural GABA_AR and GlyR as well as muscular ClC-1 gives rise to hyperpolarization (Fig. 1A), thereby stabilizing or suppressing their excitability. In contrast, activation of CaCC and GABA_AR/GlyR brings about depolarization (Fig. 1B) in smooth muscle cells and immature neurons, respectively; this triggers or enhances their excitability, because Cl⁻ is normally above the electrochemical equilibrium in these NKCC-dominated cells (Chipperfield and Harper, 2000; Blaesse et al., 2009; Payne, 2009).

In most epithelial cells in which Cl⁻ is maintained at a concentration higher than the equilibrium value by the predominant operation of NKCC (Russell, 2000), opening of anion channels results in efflux of Cl⁻ out of cells (Fig. 1B). Thus, CFTR and CaCC are involved in Cl⁻ secretion or absorption, depending on their location on the apical (luminal) or basolateral (serosal) membrane, respectively, in many epithelial tissues, including the intestinal and airway tracts. In addition, ClC-K2 is involved in Cl⁻ reabsorption in the thick ascending limb of the loop of Henle in the kidney.

C. Volume-Controlling Action of Anion Channels

The most important secondary physiologic action of anion channel opening is to regulate or change the cell volume by triggering KCl flux, because K⁺ conductance exceeds Cl⁻ conductance under resting conditions in most cells. Regulation of cell volume is fundamental to the survival and function of animal cells under both physiologic and pathophysiologic conditions (for reviews, see Lang, 1998; Okada, 1998b, 2004; Hoffmann and Pedersen, 2006; Hoffmann et al., 2009). Among the three types of VAACs, VSOR and Maxi-Cl are involved in cell volume regulation after osmotic cell swelling and thus

may be referred to as volume-regulatory anion channels (Table 1). VSOR is the most typical swelling-activated anion channel in most animal cells and plays an effective role in providing a pathway for anion efflux during the regulatory volume decrease (RVD) after cell swelling (for reviews, see Okada and Hazama, 1989; Okada, 1997). Maxi-Cl is also involved in the RVD process directly by providing the volume-regulatory pathway for anion efflux (see Okada et al., 2009b) or indirectly by releasing ATP, which stimulates P2Y receptors in an autocrine fashion (Kurbannazarova et al., 2011; Islam et al., 2012). The cardiac isoform of CFTR may be involved in the RVD process in cardiac myocytes under special conditions in which β -adrenergic receptors are stimulated (Wang et al., 1997). On the contrary, some other types of anion channels, such as CaCC, ASOR, and CFTR (called VCACs; Table 1), may facultatively or accidentally lead to changes in cell volume. This article focuses on the properties, molecular identities, functions, and pharmacology of these VAACs and VCACs.

II. Volume-Activated Anion Channels

VAACs are directly, but not solely, activated by cell swelling. For instance, activation of ClC-2, VSOR, and Maxi-Cl is also brought about by acidic pH, reactive oxygen species (ROS), and membrane patch excision, respectively, even in the absence of a volume increase. All VAACs are not necessarily volume regulatory, because ClC-2 is not involved in the RVD process (Bond et al., 1998), whereas VSOR and Maxi-Cl exert volume-regulatory actions under normal conditions (Okada, 1997; Sabirov and Okada, 2004a). Under apoptotic conditions, however, VSOR activity leads to sustained cell shrinkage, called an apoptotic volume decrease (AVD) (Maeno et al., 2000; Shimizu et al.,

2004), without attaining volume regulation. These VAACs show distinguishable voltage dependence from each other; that is, ClC-2, VSOR, and Maxi-Cl exhibit inward, outward, and no rectification, respectively (Okada et al., 2009b).

A. ClC-2-Type Chloride Channel

The ClC-2 anion channel was cloned by Jentsch's group (Thiemann et al., 1992) and belongs to the gene family of ClC channel/transporter, which consists of nine different members in mammals (Jentsch et al., 2002). ClC-2 is ubiquitously expressed and activated not only by cell swelling (Gründer et al., 1992) but also by strong membrane hyperpolarization (Thiemann et al., 1992) and by extracellular acidification (Pusch and Jentsch, 1994; Jordt and Jentsch, 1997). The channel is dependent on voltage and is inactive at normal resting potentials that are more positive than -90 mV (Thiemann et al., 1992); however, ClC-2 becomes activated at physiologic membrane potentials after cell swelling (Gründer et al., 1992). The voltage dependence of this channel is also characterized by inward rectification and inactivation of the tail macroscopic current upon a depolarizing pulse applied after hyperpolarization (Fahlke, 2001). ClC-2 single-channel conductance is small (2 to 3 pS) (Weinreich and Jentsch, 2001), and its anion selectivity is a type of strong electric field (Eisenman sequence IV) with a permeability sequence of $\text{Cl}^- > \text{Br}^- > \text{I}^-$ (Thiemann et al., 1992; Jordt and Jentsch, 1997). Activation of ClC-2 does not depend on intracellular ATP (Park et al., 1998; Rutledge et al., 2002), which is in contrast to the intracellular ATP dependence of VSOR (for review, see Okada, 1997).

Thus far, ClC-2 is suggested to be involved in a number of physiologic functions, including colonic Cl^- transport (Catalán et al., 2002; Lipecka et al., 2002), gastric acid secretion (Malinowska et al., 1995), GABA_AR-mediated inhibitory synaptic input (Smith et al., 1995) by controlling the $[\text{Cl}^-]_i$ level in neurons (Staley et al., 1996; Földy et al., 2010), lung development (Murray et al., 1995), and early nephrogenesis (Huber et al., 1998). Nonetheless, ClC-2-deficient mice were found to exhibit neither seizure nor prominent abnormalities in the development of the lung and kidney as well as in epithelial Cl^- transport and acid secretion, apart from degeneration of the retina and testis (Bösl et al., 2001).

Since studies showed that ClC-2 was activated by hypotonicity-induced cell swelling in a number of cell types, including *Xenopus* oocytes transfected with ClC-2 (Gründer et al., 1992; Jordt and Jentsch, 1997; Furukawa et al., 1998), insect Sf9 cells transfected with ClC-2 (Xiong et al., 1999), and native mouse atrial and ventricular myocytes (Duan et al., 2000), it was expected to play a role in cell volume regulation after swelling (RVD). The data suggesting such a volume-regulatory role of ClC-2 were reported in nonmammalian

ClC-2-transfected cells (Furukawa et al., 1998; Xiong et al., 1999) and in native mammalian cells (Roman et al., 2001). However, studies in *Xenopus* oocytes (Furukawa et al., 1998) and Sf9 cells (Xiong et al., 1999) were conducted in nonmammalian over-expression systems, and no direct evidence for RVD was shown in the former study. Although another study was performed in nontransfected rat hepatoma tissue culture cells (Roman et al., 2001), the conclusions were merely based on indirect observations using anti-ClC-2 antibodies. Moreover, the property of swelling-activated Cl^- currents shown by Roman et al. (2001) looks completely distinct from inward-rectifier ClC-2, exhibiting mild outward rectification similar to that of VSOR currents. In addition, the possibility that ClC-2 is involved in the RVD process was ruled out by a study performed in native human colonic T84 cells (Bond et al., 1998) on the basis of findings showing that a specific blocker of ClC-2, Cd^{2+} , failed to affect the RVD and that swelling-induced augmentation of endogenous inward-rectifier ClC-2 currents occurred only after prior activation by hyperpolarizing voltages. Furthermore, the involvement of ClC-2 in RVD was refuted by observations that parotid acinar cells from *Clcn2* knockout mice showed RVD with similar efficiency to that for wild-type littermates (Nehrke et al., 2002). Taken together, we conclude that ClC-2 is activated by cell swelling but is not a major contributor to the RVD process.

B. Volume-Sensitive Outwardly Rectifying Anion Channel

VSOR (also called VRAC) is ubiquitously expressed and directly activated by cell swelling induced by decreased extracellular or increased intracellular osmolality. In 1988, two groups functionally discovered this channel independently in human epithelial cells (Hazama and Okada, 1988) and in human lymphoid cells (Cahalan and Lewis, 1988). The single-channel event with an intermediate unitary conductance (10–90 pS), which could sometimes be observed during recording of the whole-cell current under nonswollen conditions, was suggested to be responsible for the swelling-induced macroscopic current in human epithelial T84 cells, based on similarities in their voltage dependence (Worrell et al., 1989). Using a double patch-clamp technique under swollen conditions, direct evidence for the causal involvement of intermediate single-channel events in macroscopic VSOR currents was provided by simultaneous recordings of the single-channel and whole-cell currents in single human epithelial Intestine 407 cells (Okada et al., 1994b; Petersen et al., 1994) and mouse cortical neurons (Inoue and Okada, 2007; Okada et al., 2009b).

VSOR activity plays a primary role in cell volume regulation after swelling, called RVD, by serving as the volume-regulatory Cl^- exit pathway (Hazama and Okada, 1988; Okada and Hazama, 1989; Kubo and

Okada, 1992). As a natural consequence, VSOR activity is secondarily involved in a variety of physiologic cell functions that are inevitably associated with whole or local cell swelling, such as cell proliferation or mitosis, cell migration, and cell differentiation (for reviews, see Eggermont et al., 2001; Hoffmann et al., 2009; Akita and Okada, 2014; Pedersen et al., 2015, 2016). In addition, VSOR is known to serve as a pathway for swelling-induced efflux of organic signaling solutes such as glutamate (Kimelberg et al., 1990; Strange et al., 1996; Kirk, 1997; Liu et al., 2006, 2009; Okada et al., 2009b; Akita and Okada, 2014; Hyzinski-García et al., 2014; Planells-Cases et al., 2015; Lutter et al., 2017; Schober et al., 2017). Furthermore, VSOR is involved in the induction of apoptosis (Maeno et al., 2000; Shimizu et al., 2004), as well as to anticancer drug resistance (Lee et al., 2007; Shimizu et al., 2008; Sørensen et al., 2016) and transport (Planells-Cases et al., 2015).

1. Phenotypical Properties. A large number of studies clarified the phenotypical properties of VSOR (see reviews by Okada et al., 1994a; Strange et al., 1996; Nilius et al., 1997a; Okada, 1997, 2006; Shimizu et al., 2008; Akita and Okada, 2014) as follows: volume sensitivity, mild outward rectification (Fig. 2A), voltage- and time-dependent inactivation (Fig. 2, A and B), intermediate single-channel conductance (Fig. 2B), low-field-strength anion selectivity with Eisenman sequence type I ($I^- > Br^- > Cl^- > F^-$), nonhydrolytic requirement of intracellular ATP, sensitivity to intracellular free Mg^{2+} , and open-channel block by extracellular ATP. The mild outward rectification of VSOR is its most characteristic biophysical property, which is distinct from two other VAACs [inward-rectifier ClC-2 and ohmic (I - V linear) Maxi-Cl] and from three other VCACs [ohmic CFTR, sharp outward-rectifier CaCC, and ASOR]. The intermediate unitary conductance of VSOR is distinct from the small unitary conductance of ClC-2 (2 to 3 pS), CFTR (6–12 pS), CaCC (0.45–1.2 pS), and ASOR (4–7 pS) and from the large unitary conductance of Maxi-Cl (300–500 pS). Recent nonelectrolyte partition studies revealed that the cut-off radius of the narrowest constriction inside the VSOR pore is 0.63 nm (Ternovsky et al., 2004), which is much smaller than that inside the Maxi-Cl pore (approximately 1.3 nm; Sabirov and Okada, 2004b) and larger than that inside the CFTR pore (approximately 0.27 nm; Linsdell et al., 1997). The low-field-strength halide anion selectivity of VSOR is similar to Maxi-Cl, CaCC, and ASOR but distinct from the higher-field anion selectivity of ClC-2 ($Cl^- > Br^- > I^- > F^-$: Eisenman type IV) and CFTR ($Br^- > Cl^- > I^- > F^-$: Eisenman type III). The voltage-dependent blocking of outward (not inward) VSOR currents by extracellular ATP at > 0.1 mM is one of the unique characteristics of VSOR (Jackson and Strange, 1995; Tsumura et al., 1996). This is due to plugging of the anion-conducting pathway of VSOR by ATP, because the VSOR pore radius is nearly the

same as the effective radius of ATP (approximately 0.6 nm).

The dependence of VSOR on intracellular ATP is its most important physiologic property (Jackson et al., 1994; Oiki et al., 1994), which also distinguishes it from ClC-2 and Maxi-Cl, both of which are rather inhibited by intracellular ATP (Rutledge et al., 2002; Sabirov and Okada, 2009). Under constant intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) conditions, instantaneous Cl^- currents recorded at +40 mV in human epithelial Intestine 407 cells increased with the increasing intracellular free ATP concentration ($[ATP]_i$), as shown in Fig. 3A. The half-maximal effective concentration (EC_{50}) for ATP was very low (0.014 mM at 1 mM free Mg^{2+}). Even when intracellular Mg^{2+} ions were largely chelated by EDTA (5 mM), VSOR currents were not attenuated in the presence of intracellular ATP (Fig. 3B; at $[Mg^{2+}]_i = 10^{-5}$ mM). Therefore, it appears that free ATP, but not Mg-bound ATP, activates the channel, and the role of free ATP is not mediated by its hydrolysis but rather presumably by its binding to some functional VSOR component. This result is consistent with previous observations that a nonhydrolyzable ATP analog (β,γ -imidoadenosine 5'-triphosphate) and a slowly hydrolyzable ATP analog (adenosine 5'-*o*-13-thiotriphosphate) did not abolish but rather could substitute, at least in part, for ATP in human epithelial Intestine 407 cells (Oiki et al., 1994), as observed in other cell types (Díaz et al., 1993; Jackson et al., 1994).

VSOR activity is known to be suppressed by an increase in cytosolic free Mg^{2+} (Oiki et al., 1994; Okada, 1997). In the presence of a constant $[ATP]_i$ (0.1 mM), the current amplitude was decreased (as shown in Fig. 3B), in a concentration-dependent manner, by increases in $[Mg^{2+}]_i$ or by concomitant increases in the concentration of the intracellular Mg-ATP complex. If Mg-ATP is an inhibitory factor, the inhibition curve plotted on the $[Mg^{2+}]_i$ axis must shift to the left (toward lower $[Mg^{2+}]_i$) by elevation of $[ATP]_i$, because the concentration of the intracellular Mg-ATP complex is increased by elevation of $[ATP]_i$ at fixed $[Mg^{2+}]_i$ according to the law of mass action. On the contrary, increased $[ATP]_i$ brought about a shift of the $[Mg^{2+}]_i$ -inhibition curve to the right, in the opposite direction (Fig. 3B). Thus, it appears that the channel activity is suppressed by free Mg^{2+} ions but not by the ATP-bound form of Mg. The half-maximal inhibitory concentration (IC_{50}) for free Mg^{2+} was 0.32 mM at 0.01 mM $[ATP]_i$ and 1.9 mM at 0.1 mM $[ATP]_i$. The normalized peak I - V relationship was little affected by altering $[Mg^{2+}]_i$ (Fig. 3C). Thus, intracellular Mg^{2+} suppresses VSOR currents in a voltage-independent manner.

2. Activation Mechanisms. The mechanism of swelling-induced VSOR activation has long been discussed (for reviews, see Nilius et al., 1997a; Okada, 1997; Hoffmann et al., 2009; Okada et al., 2009a; Akita and Okada, 2014). Although many fluid-soluble signals

Fig. 2. VSOR and Maxi-Cl currents in human monocytic U937 cells. (A) Macroscopic whole-cell VSOR currents recorded from U937 cells after osmotic swelling (left) and the corresponding current-to-voltage relationship (right). Experimental conditions, other than the cell line used, are the same as reported previously (Sabirov et al., 2001; Kurbannazarova et al., 2011). (B) Single VSOR currents recorded from cell-attached patches on preswollen cells. Holding membrane potentials are indicated on the left of each trace. The right panel shows the unitary current-voltage relationship. The calculated slope conductance is 84.5 ± 5.8 and 14.7 ± 2.4 pS for outward and inward currents, respectively. Experimental conditions, other than the cell line used, are the same as reported previously (Ternovsky et al., 2004; Kurbannazarova et al., 2011) with 100 mM TEACl-pipette and 100 mM KCl-bath solutions. (C) Activation of Maxi-Cl currents in U937 cells. (Left) Voltage- and time-dependent inactivation of the steady-state membrane current as observed in excised macropatches in response to voltage pulses (500 milliseconds) from a holding potential at 0 to ± 50 mV in 10-mV increments. The pipette and bath solutions were normal Ringer's solution. (Right) Single-channel I-V relationships for maxi-anion channel events recorded. Each symbol represents the mean $\pm$ S.E. (vertical bar). The solid line is a linear fit with a slope conductance of 445.3 ± 9.6 pS. Experimental conditions, other than the cell line used, are the same as reported previously (Islam et al., 2012).

including a number of kinases and small G proteins have been suggested to be involved in the mechanism, they have only partial or permissive effects (Okada et al., 2009a).

Direct activation of VSOR by increased membrane stretch or tension can be excluded from the possible mechanism in light of the following observations. First, cell swelling does not necessarily bring about membrane stretch, because the cell membrane has sufficient reserves (namely, infoldings or invaginations) to allow for several-fold volume increases without producing

plasmalemmal stretch (Levitan and Garber, 1996; Okada, 1997, 2004; Groulx et al., 2006; Pangrsic et al., 2006). Second, in the cell-attached configuration, membrane stretch induced by applying negative pressure via a patch pipette failed to activate VSOR on nonswollen Intestine 407 cells and did not affect single-channel activity on the swollen cells (Okada, 1997). A lack of association between stretch-activated and swelling-activated Cl^- currents was also demonstrated in human hepatocellular carcinoma cells (Mao et al., 2011) and in human neutrophils (Behe et al., 2017).

Fig. 3. Cl⁻ current amplitudes plotted against [ATP]_i, [Mg²⁺]_i, and voltages. Each symbol represents the mean current of four different Intestine 407 cells. Vertical bars represent the S.E. (A) [ATP]_i dependence of the peak Cl⁻ currents measured at +40 mV at a fixed [Mg²⁺]_i (1 mM). Data points were fitted by the following equation: $I = I_{\max}([ATP]_i/EC_{50})/(1 + ([ATP]_i/EC_{50}))$. (B) [Mg²⁺]_i dependence of the peak Cl⁻ currents measured at +40 mV at a fixed [ATP]_i (0.1 or 0.01 mM). Data points were fitted by the following equation: $I = I_{\max}/(1 + ([Mg^{2+}]_i/IC_{50}))$. (C) Current-voltage relationships. Normalized peak Cl⁻ currents ($I_V/I_{+40\text{-mV}}$) were measured at different [Mg²⁺]_i and fixed [ATP]_i. Mg²⁺ did not change the reversal potential, indicating that the Mg²⁺ effect is independent of voltage and that Mg²⁺ cannot permeate the channel. Experimental conditions are the same as reported previously (Oiki et al., 1994).

Instead of membrane tension, unfolding of the membrane invaginations is likely important for channel activation, because the single-channel event of VSOR could be recorded only when the patch pipette was attached to the preswollen cell but not when giga-sealed patches had been formed before the cell was rendered swollen (Okada, 1997, 1998a) and also because the density of whole-cell VSOR currents increased in proportion to the square of the cell diameter (namely, the outer surface area of cell) (Miwa et al., 1997; Okada, 1997; Morishima et al., 2000). Since the swelling-induced activation of VSOR is attained in a manner independent of exocytotic insertion (Okada et al., 1992), VSOR proteins may exist in a largely inactivated state on the plasma membrane prior to a hypotonic challenge and become activated by cell swelling or membrane unfolding. Assuming that the preexisting VSOR protein may readily interact with other proteins (e.g., cytoskeletal or actin-binding proteins) in such a confined space within infoldings filled with the actin-based meshwork, Okada (1997) put forward the “unfolding-induced protein-protein interaction hypothesis” for the VSOR activation mechanism, referred to here as the “type A mechanism” (Fig. 4).

Our more recent work (Ando-Akatsuka et al., 2012) confirmed this hypothesis by the following step-by-step studies in human embryonic kidney 293 T (HEK293T) cells. First, we searched a cytoskeleton-binding protein, which becomes associated with the cell membrane upon cell swelling; we then found that it is an actin-binding protein, α -actinin-4 (ACTN4), which is known to be present ubiquitously in nonmuscle cells (Sjöblom et al., 2008). Second, ABCF2, a cytosolic member of the ATP-binding cassette (ABC) transporter superfamily, was identified as the binding partner of ACTN4 by protein overlay assays combined with proteomics approaches. Third, overexpression and small interfering RNA (siRNA)-mediated downregulation of ABCF2

expression reduced and enhanced VSOR activity, respectively. Thus, we conclude that swelling-induced VSOR activation is attained by membrane unfolding-induced protein-protein interactions between ACTN4 and ABCF2, which prevents ABCF2 from suppressing VSOR activity presumably by releasing ABCF2 from VSOR protein (Fig. 4, type A mechanism). Because the submembrane actin ring is disrupted by cell swelling (Levitani et al., 1995; Morán et al., 1996; Pedersen et al., 1999; Carton et al., 2003; Erickson et al., 2003; Pritchard and Guilak, 2004), ACTN4 might be released from actin, mobilized onto the membrane-rich fraction, and thereby become available for the interaction with ABCF2 (Ando-Akatsuka et al., 2012). Since another ABC protein (CFTR) is known to downregulate VSOR activity (Vennekens et al., 1999; Ando-Akatsuka et al., 2002), there is a possibility that VSOR interacts with ABC proteins other than ABCF2.

Activation of VSOR is provoked even in the absence of cell swelling or membrane unfolding under conditions in which the ROS level is increased (Fig. 4, type B mechanism). Swelling-independent, H₂O₂-induced activation of VSOR was originally found independently in 2004 by three groups (Browe and Baumgarten, 2004; Shimizu et al., 2004; Varela et al., 2004) and was confirmed by subsequent studies (Wang et al., 2005; Jiao et al., 2006; Varela et al., 2007; Harrigan et al., 2008; Liu et al., 2009; Deng et al., 2010a,b; Crutzen et al., 2012; Holm et al., 2013; Shen et al., 2014; Xia et al., 2016; Wang et al., 2017a,b). In addition, for the first time, VSOR per se was identified as the anion channel responsible for the Cl⁻ efflux causing apoptotic cell shrinkage, called AVD (Maeno et al., 2000), which was observed after stimulation with an intrinsic mitochondrion-mediated apoptosis inducer (staurosporine) due to increased ROS production via NAD(P)H oxidase (NOX) (Shimizu et al., 2004) (Fig. 4, type B mechanism). Then, ROS-mediated VSOR activation

Fig. 4. Dual mechanisms for VSOR activation. The bottom half represents the membrane unfolding-induced (type A) mechanism, which takes place upon cell swelling, and the top half represents the oxidation-induced (type B) mechanism, which takes place upon receptor stimulation (see the text for details and other abbreviations). BKR, bradykinin receptor; DAG, diacylglycerol; Gq, heterotrimeric G protein that activates phospholipase C; IP₃, inositol trisphosphate; mGluR, metabotropic glutamate receptor; P2YR, P2Y purinergic receptor; PKC, protein kinase C; PLC, phospholipase C; ROC, receptor-operated channel; SOC, store-operated channel.

was observed without cell swelling under stimulation with a variety of apoptosis inducers, including an endoplasmic reticulum (ER) stress-mediated apoptosis inducer (tunicamycin; Shen et al., 2014), a stress-induced apoptosis inducer (ceramide; Raucci et al., 2010), and an anticancer drug (doxorubicin; Kumagai et al., 2012). An extrinsic death receptor-mediated apoptosis inducer, tumor necrosis factor α , was also reported to induce ROS-mediated (oxidation-induced) VSOR activation in mouse proximal convoluted tubule cells (l'Hoste et al., 2010) and mouse aortic smooth muscle cells (Matsuda et al., 2010b), although not in human HeLa cells (Shimizu et al., 2004). Baumgarten's group showed that oxidation-induced VSOR activation in cardiac myocytes is brought about by stimulation of angiotensin II receptor type 1 (Browe and Baumgarten, 2004; Ren et al., 2008), the epidermal growth factor receptor (Browe and Baumgarten, 2006), and the endothelin A receptor (Deng et al., 2010a) in rabbit ventricular myocytes. We also showed that ROS-mediated VSOR activation in astrocytes under isotonic conditions can be elicited by stimulation of Gq protein-coupled receptors such as the bradykinin B₂ receptor (Liu et al., 2009; Akita and Okada, 2011), purinergic P2Y receptors (Akita et al., 2011), and metabotropic glutamate receptor 5 (Akita and Okada, 2014). As summarized in Fig. 4 (type B mechanism), we revealed that this receptor-mediated VSOR activation is induced by NOX-mediated ROS production and is controlled via high-concentration regions of intracellular Ca²⁺ (called "Ca²⁺ nanodomains") in the immediate vicinity of open Ca²⁺-permeable channels such as store- and receptor-operated channels (Akita and Okada, 2011; Akita et al., 2011). Such NOX-mediated ROS production controlled by Ca²⁺ nanodomains may also account for the mechanism of adenosine-induced, purinergic P1 receptor-mediated activation of VSOR currents in kidney tubular cells, because this was found to be coupled to Ca²⁺ entry through Ca²⁺-permeable channels sensitive to La³⁺ and

Gd³⁺ (Rubera et al., 2001). In this regard, it is worth noting that leucine-rich repeats (LRRs) containing 8A (LRR8A), which was recently identified as one of the core components of VSOR (Qiu et al., 2014; Voss et al., 2014), was found to colocalize and interact with NOX1 (Choi et al., 2016).

Since membrane unfolding- and oxidation-induced activation was shown to be additive under certain conditions (Gradogna et al., 2017), type A and type B mechanisms would be independent of each other. When cells respond to hypo-osmotic stress not only with osmotic cell swelling but also with ATP release, VSOR activation can dually be attained by the Ca²⁺-independent, membrane unfolding-induced type A mechanism and by the Ca²⁺ nanodomain-dependent, oxidation-induced type B mechanism (Fig. 4). As shown in Fig. 4, in fact, the VSOR current activated upon osmotic swelling was estimated to be composed of approximately 40% for the oxidation-induced, Ca²⁺ nanodomain-mediated component and another approximately 60% for the unfolding-induced, Ca²⁺-independent component in mouse astrocytes (Akita et al., 2011).

There is a possibility that even in the oxidation-induced (type B) mechanism for VSOR activation, ABCF2 is released from the VSOR protein and then binds to ACTN4. In fact, as shown in Fig. 5, exposure to H₂O₂ facilitated ACTN4 mobilization onto the radioimmunoprecipitation assay buffer-insoluble membrane- and cytoskeleton-rich (10,000g pellet) fraction (Fig. 5, A and B) and then promoted the ABCF2-ACTN4 interaction, which was monitored by coimmunoprecipitation, in the radioimmunoprecipitation assay buffer-insoluble fraction (Fig. 5, C and D). Thus, it appears that the dissociation of ABCF2 from VSOR is the final common step for dual mechanisms, which include membrane unfolding- and oxidation-induced VSOR activation.

The VSOR current was activated without visible cell swelling by intracellular dialysis with guanosine

Fig. 5. Effects of H₂O₂ stimulation on the subcellular localization of ACTN4 and molecular interaction between ACTN4 and ABCF2. (A and B) Accumulation of ACTN4, but not ABCF2, in the RIPA-insoluble fraction with 0.5 mM H₂O₂ stimulation. Five micrograms of RIPA-soluble and RIPA-insoluble fractions of the 100,000g pellet prepared from the cells transiently overexpressing ACTN4/FLAG and ABCF2/HA were subjected to SDS-PAGE and immunoblotting with anti-FLAG M2 mAb and anti-HA and anti-caveolin-1 antibodies. (A) Immunoblots. ACTN4/FLAG, ABCF2/HA, and caveolin-1 were detected. Molecular weight markers (in kilodaltons) are indicated on the left. (B) Densitometric analysis of the immunoblot data shown in (A). The ratio of the ACTN4/FLAG contents (normalized with caveolin-1 content) in the presence of 0.5 mM H₂O₂ to that in the absence of 0.5 mM H₂O₂ is plotted. **P* < 0.05 (significant difference between the data from cells with or without H₂O₂ stimulation). (C and D) H₂O₂ stimulation enhances the interaction between ACTN4 and ABCF2 in the RIPA-insoluble fraction. (C) RIPA-soluble and RIPA-insoluble proteins from the 100,000g pellets were immunoprecipitated using anti-FLAG M2 mAb and were immunoblotted with anti-FLAG mAb and anti-HA antibody (right). Transfected ACTN4/FLAG and ABCF2/HA were shown in the input (left). (D) Densitometric analysis of the immunoblot data shown in (C). The ratio of the coprecipitated ABCF2/HA content (normalized with the precipitated ACTN4/FLAG content) with H₂O₂ stimulation (black columns) to that of control (white columns) is plotted. **P* < 0.05 (significant difference between the data from cells with or without H₂O₂ stimulation). Experimental conditions are the same as reported previously (Ando-Akatsuka et al., 2012). HA, hemagglutinin; IB, immunoblotted; IP, immunoprecipitated; mAb, monoclonal antibody; RIPA, radioimmunoprecipitation assay buffer.

5'-3-*O*-(thio)triphosphate (GTP γ S) (Doroshenko et al., 1991; Doroshenko and Neher, 1992; Nilius et al., 1994a; Shen et al., 1996; Mitchell et al., 1997b; Estevez et al., 2001) or by a reduction in intracellular ionic strength (Cannon et al., 1998; Nilius et al., 1998; Voets et al., 1999; Sabirov et al., 2000). However, it is not known how these maneuvers lead to activation of VSOR currents. Since intracellular introduction of GTP γ S may make a great impact on G protein-coupled receptors and small G proteins, especially Rho family members, which regulate actin cytoskeleton dynamics (Pedersen et al., 2001), GTP γ S may thereby affect both type A and type B mechanisms for VSOR activation. Reduced ionic strength should increase the surface potential on a number of intracellular proteins, which possess multivalent negative charges and may thereby affect the dual mechanisms of VSOR activation. Since F-actin is known

to be disrupted at lower ionic strength (Senger and Goldmann, 1995; Miyata et al., 1997), it is conceived that swelling-induced VSOR activation by reduced ionic strength is mediated by actin disassembly, which was reported to enhance VSOR activation (Levitan et al., 1995; Okada, 1997). In this regard, VSOR activation was actually found to be induced without cell swelling or even in shrunken cells under conditions of very low ionic strength (Cannon et al., 1998). However, it must be noted that swelling-induced activation of VSOR is not solely accounted for by reduced ionic strength, because VSOR can be robustly activated by inflating cells by forcing isotonic fluid with identical ionic strength into them through the patch pipette under the whole-cell configuration (Hagiwara et al., 1992; Doroshenko, 1998; Best and Brown, 2009). Recently, VSOR-like anion channel activity was found to be activated by reducing

ionic strength when purified LRRC8A proteins were reconstituted together with LRRC8D or LRRC8E (Syeda et al., 2016). There is a possibility that this channel activation in response to reduced ionic strength was caused by modification of charge-mediated interaction between the LRR domains of LRRC8A and 8D or 8E. However, this channel activity is somewhat different from the native VSOR activity, in light of its small single-channel conductance (approximately 8 pS), independence of intracellular ATP, and lack of inactivation kinetics. Such nonspecific activating effects of reduced ionic strength were also found on non-VSOR-type anionic currents residually observed in neutrophils isolated from *ebo/ebo* mice in which LRRC8A truncation mutants naturally occur (Behe et al., 2017).

The swelling-induced VSOR current was potentiated by cytosolic cAMP produced by stimulation of Gs-coupled receptors such as the Ca^{2+} -sensing receptor (Shimizu et al., 2000) and the vasopressin V_2 receptor (Sato et al., 2011). Although the mechanism by which cAMP potentiates VSOR activity is not known precisely, there is a possibility that cAMP modulates cytoskeleton rearrangement, thereby indirectly augmenting VSOR activity by affecting the ACTN4-ABCF2 interaction (Fig. 4).

3. Molecular Identity. The molecular identification of VSOR has passed through various vicissitudes of fortune. For the VSOR protein, at least five candidates, including P-glycoprotein, pI_{Cln} , ClC-3 , bestrophin-1 (Best1), and some transmembrane TMEM16/anoctamin (ANO) members (particularly TMEM16F/ANO6), have been proposed thus far but were dismissed, almost one after another, by successive works performed in a large number of laboratories (see reviews by Nilius et al., 1997b; Okada, 1997; Nilius and Droogmans, 2001; Jentsch et al., 2002; Okada et al., 2009b, 2018; Pedersen et al., 2016). One of the essential components of VSOR was finally discovered in 2014 (Qiu et al., 2014; Voss et al., 2014).

Using an unbiased genome-wide siRNA screening method with an I^- -sensitive fluorescence-based assay, two independent groups simultaneously reported that a membrane-spanning protein (LRRC8A) acts as an essential component of VSOR molecular identity in human cell lines and T lymphocytes (Qiu et al., 2014; Voss et al., 2014). Both groups convincingly showed that knockdown of human LRRC8A (hLRRC8A) abrogated swelling-induced VSOR currents and taurine release, thereby suppressing RVD in several human cell lines. In addition, the authors demonstrated that transfection of hLRRC8A cDNA rescued VSOR activity in stable LRRC8A knockdown cells, indicating that hLRRC8A plays an essential role in human VSOR activity. Subsequently, knockdown of mouse LRRC8A was also demonstrated to greatly reduce VSOR activity in a mouse C127 cell line (Okada et al., 2017) and in primary cultured mouse nodose ganglia neurons (Wang et al.,

2017b). More recently, mouse VSOR activity was found to be drastically diminished in T cells from *Lrrc8a*^{-/-} mice and *ebo/ebo* mice with a *Lrrc8a* frameshift mutation (Platt et al., 2017) and in neutrophils from *ebo/ebo* mice (Behe et al., 2017). Thus, it is evident that LRRC8A is an essential core component of VSOR not only in human cells but also in rodent cells. This notion was indirectly supported by the following three studies on swelling-induced osmolyte release, which is shown to be mediated via VSOR. First, siRNA-mediated knockdown of LRRC8A reduced the swelling-induced release of aspartate, glutamate, and taurine from rat astrocytes (Hyzinski-García et al., 2014). Second, a reduction in swelling-induced taurine release in cisplatin-resistant human ovarian cancer A2780 cells correlated with reduced expression of LRRC8A (Sørensen et al., 2014). Third, the swelling-induced release of neurotransmitters including glutamate, D-aspartate, D-lysine, D-serine, GABA, and taurine was observed in wild-type human embryonic kidney 293 (HEK293) cells but was abolished in *LRRC8A*^{-/-} HEK293 cells (Lutter et al., 2017). Such a role of LRRC8A in anion channel formation appears to be specific to VSOR activity, because no significant effect of LRRC8A knockdown was observed on ASOR, Maxi-Cl, CaCC, and CFTR currents in mouse C127 cells (Okada et al., 2017).

The LRRC8 family consists of five members (LRRC8A–8E), and LRRC8D was shown to directly interact with LRRC8A, 8B, and 8C by immunoprecipitation studies coupled to mass spectrometry (Lee et al., 2014). In addition, sequential coimmunoprecipitation studies provided evidence for the interaction between LRRC8A, LRRC8C, and LRRC8E (Lutter et al., 2017). Hence, there is a possibility that LRRC8A forms heteromers with four other LRRC8 members, thereby participating in VSOR activity (Stauber, 2015). In fact, VSOR currents in human HCT116 and HEK293 cells lacking all five LRRC8 genes were rescued by expressing LRRC8A together with LRRC8C or 8E (Voss et al., 2014). Also, endogenous VSOR activity in HeLa cells was abolished by quadruple knockdown of LRRC8B–8E (Sato-Numata et al., 2017). These data suggest that the VSOR heteromer consists of LRRC8A together with LRRC8C, 8D, and/or 8E. Supporting this hypothesis, the swelling-induced release of aspartate from rat astrocytes was largely suppressed by not only double knockdown of LRRC8C and 8D but also by double knockdown of LRRC8A and 8E (Schober et al., 2017). Accumulating evidence has shown that LRRC8B may not be requisitely involved in VSOR activity, as follows. First, when LRRC8B was transfected together with LRRC8A in HCT116 cells with disruption of all five LRRC8 genes (*LRRC8A*^{-/-} cells), VSOR activity was not restored under hypotonic conditions (Voss et al., 2014). Second, when *Xenopus* oocytes, which virtually lack all LRRC8 genes and endogenous VSOR activity, were coinjected with LRRC8A with 8B or that of

LRRC8A tagged with fluorescent proteins (8A*) and 8B*, hypotonic stimulation failed to activate VSOR currents, although coinjection of LRRC8A plus 8E/8D or of 8A* plus 8C*/8D*/8E* produced VSOR activity (Gaitán-Peñas et al., 2016). In addition, VSOR activity was not induced by coinjection of 8A* with 8B* even after application of a membrane-permeable oxidizing agent, whereas VSOR currents induced by coinjection of 8A* plus 8C*/8D*/8E* were, in contrast, greatly affected by oxidation (Gradogna et al., 2017). Third, triple knockdown of LRRC8C, 8D, and 8E abolished endogenous VSOR activity in HeLa cells, which endogenously express all five LRRC8 genes (Sato-Numata et al., 2017). Fourth, disruption of LRRC8C, 8D, and 8E abolished endogenous VSOR activity in HEK293 cells; thus, the remaining LRRC8A and 8B failed to maintain VSOR activity, whereas LRRC8A and 8C/8D/8E could largely express VSOR activity (Lutter et al., 2017). Thus, it is likely that LRRC8B plays some role other than a VSOR component. In fact, LRRC8B was recently reported to be localized in the ER and was associated with ER calcium leakage in HEK293 cells (Ghosh et al., 2017).

By qualitatively evaluating the ratio of currents at the end to beginning of a 2-second pulse ($I_{2\text{sec}}/I_{\text{max}}$), Jentsch's group observed that the VSOR currents in *LRRC8*^{-/-} HCT116 cells coexpressing LRRC8A with LRRC8E exhibited faster inactivation kinetics upon application of depolarizing pulses, whereas those of the cells coexpressing LRRC8A with LRRC8C appeared to inactivate much more slowly (Voss et al., 2014). Thereafter, they showed that the C-terminal part of the first extracellular loop of LRRC8E determines the inactivation kinetics of VSOR currents in this cell line (Ullrich et al., 2016). These data suggest that LRRC8E per se or its associated subcomponent represents the inactivation gate or its accelerating factor, whereas LRRC8C per se or its associated subcomponent acts as the decelerating factor. In contrast to HCT116 cells, inactivation kinetics more accurately evaluated by the half-maximal inactivation time in HeLa cells were significantly hastened by siRNA-mediated knockdown of hLRRC8A but not of other LRRC8 members, even including LRRC8E and 8C, whereas the magnitude of VSOR currents was, at the same time, significantly suppressed by knockdown of LRRC8A but was not affected by other LRRC8 members (Sato-Numata et al., 2017). This fact suggests that LRRC8A per se or its associated regulatory subcomponent serves as a decelerating factor for VSOR inactivation in HeLa cells. In this cell line, knockdown of any single LRRC8 isoform also failed to retard the inactivation rate (Sato-Numata et al., 2017), suggesting that an as-yet-unidentified accelerating factor or subcomponent other than LRRC8 members is involved in the inactivation kinetics of VSOR currents.

Taken together with the data obtained by Voss et al. (2014), it is natural to deem that any LRRC8 member per se does not provide the inactivation gate for VSOR, and that some subcomponents associated with the pore-forming core components may participate in the inactivation gate or act as the accelerating and decelerating for the gate. In any case, any mutational changes in the inactivation kinetics may not provide direct evidence for an involvement of this molecule in the channel pore per se. Qiu et al. (2014) showed that VSOR activity in HeLa cells expressing the T44C mutant of LRRC8A in which threonine-44 was substituted with cysteine was strongly suppressed by a membrane-impermeable, thiol-reactive reagent sodium (2-sulfonatoethyl)methanethiosulfonate, and this mutation increased VSOR permeability to I⁻. However, the substitutions of threonine at position 44 by glutamate and arginine (T44E and T44R), which confer negative and positive charges, respectively, on this site unexpectedly had little effect on the permeability ratio of I⁻ to Cl⁻ through the channel (Qiu et al., 2014). Also, combinatory expression of charge-neutralizing mutants for K98 (K98A or K98N) of LRRC8A and for K91 (K91A or K91N) of LRRC8E failed to affect the sequence of I⁻ > Cl⁻ permeability of VSOR currents in *LRRC8*^{-/-} HCT116 cells (Ullrich et al., 2016). Furthermore, charge-reversal mutants for K98 (K98E) of LRRC8A, for K100 (K100E) of LRRC8C, and for K91 (K91E) of LRRC8E did not affect anion-to-cation selectivity but only led to a small change in the degree of I⁻ and Cl⁻ permeability while maintaining the same I⁻ > Cl⁻ permeability sequence (Ullrich et al., 2016). Thus, these observations rather suggest that T44 and K98 of LRRC8A, K100 of LRRC8C, and K91 of LRRC8E are not located at the anion-selective filter within the VSOR pore. However, different heteromeric combinations of LRRC8A together with LRRC8C, 8D, or 8E may influence VSOR pore conformation, thereby only slightly affecting the pore properties including single-channel conductance, I⁻ > Cl⁻ permeability, outward rectification, and open probability in the reconstituted system (Syeda et al., 2016). More recently, LRRC8 subunit composition was found to determine the selectivity of VSOR to organic substrates such as cisplatin and taurine (Planells-Cases et al., 2015) as well as GABA, taurine, and myo-inositol (Lutter et al., 2017). Before firmly judging these facts as evidence of pore formation by LRRC8, however, it is necessary to verify that the pathway for these neutral osmolytes is identical to that for anions.

Our recent microarray studies and Western blot analyses showed that the expression level of LRRC8A mRNA and protein in human VSOR-deficient KCP-4 cells is not much different from that in parental VSOR-rich KB cells and human VSOR-rich HEK293T, HeLa, and Intestine 407 cells (Okada et al., 2017). In addition, we noted that expression levels of LRRC8D and 8E

mRNAs in VSOR-deficient KCP-4 cells are not significantly different from the above-mentioned four other human VSOR-rich cell lines (Okada et al., 2017). Furthermore, expression levels of LRRC8B and 8C mRNAs in KCP-4 cells are rather higher than those in these VSOR-rich cell lines (Okada et al., 2018). Thus, there still remains a possibility that some molecule other than LRRC8 members serves as a main component forming the VSOR pore. In this regard, it should be noted that overexpression of LRRC8A rather gave rise to great suppression of endogenous VSOR currents in human cells (Qiu et al., 2014; Voss et al., 2014) and mouse cells (Okada et al., 2017), suggesting that excess LRRC8A expression induces a dominant negative effect caused by the formation of complexes exerting a pore-closing or pore-disrupting action (Okada et al., 2017). Moreover, LRRC8A overexpression together with LRRC8C failed to enhance endogenous VSOR currents (Voss et al., 2014). This fact may represent firm evidence that there is some as-yet-unidentified non-LRRC8 core component (X) for VSOR. In addition, anionic currents of the channels reconstituted with LRRC8A plus 8D or 8E were independent of intracellular ATP and did not exhibit voltage-dependent inactivation kinetics (Syeda et al., 2016). Thus, this missing core component is additionally required to reproduce intracellular ATP dependence and voltage-dependent inactivation kinetics, which are phenotypical characteristics of VSOR currents. In HeLa cells, knockdown of each LRRC8 member did not abolish or slow the voltage-dependent inactivation kinetics of VSOR currents (Sato-Numata et al., 2017), again suggesting that some other core component X exists that is essentially involved in or associated with the inactivation gate in this human epithelial cell line.

An involvement of NOX activity in the mechanism of VSOR activation may raise the possibility that some member of the NOX family represents the missing core component X. Supporting this hypothesis, LRRC8A (a core component of VSOR) was recently found to colocalize and interact with NOX1 in primary aortic smooth muscle cells (Choi et al., 2016). In addition, VSOR activation was suggested to be dependent in part on the assembly of an active NOX complex on the basis of suppressing effects of an inhibitor of NOX assembling, 4-(2-aminoethyl)-benzenesulfonyl fluoride, on VSOR currents in human neutrophils (Ahluwalia, 2008a). Seven members of the NOX family (NOX1–NOX5 and dual oxidases DUOX1 and DUOX2) share the capacity to transport electrons across the plasma membrane and generate superoxide and other downstream ROS (Bedard and Krause, 2007). However, microarray assays showed that expression levels of mRNAs for NOX1, NOX3, NOX4, DUOX1, and DUOX2 in VSOR-rich human epithelial cell lines (HeLa, intestine 407, KB, and HEK293T) were very low

(unpublished data). Moreover, when NOX1, NOX2, NOX4, and NOX5 were overexpressed in HEK293T cells, swelling-induced VSOR currents were not significantly affected, as shown in Fig. 6. These facts may preclude the above possibility.

The following criteria for the molecular identification of the main component of VSOR were previously proposed (Okada et al., 1998). First, transfection with the gene for the candidate protein induces swelling-activated anionic currents with characteristics identical to those of phenotypic properties of VSOR. Second, cells functionally exhibiting the VSOR current endogenously express the candidate mRNA and protein. Third, elimination of the candidate protein expression abolishes the endogenous VSOR current. Fourth, mutation of the candidate gene gives rise to significant changes in the important pore properties of the channel, such as single-channel conductance, rectification, and anion or anion/cation selectivity. A fifth criterion can also be proposed: channel activity exhibiting all of the phenotypic properties (see section II.B.1) must be reconstituted by incorporating the candidate protein(s) in the lipid bilayer. Although the essential component of VSOR has now been identified as LRRC8A plus LRRC8C/8D/8E (Voss et al., 2014), findings showing that this combinatory expression did not reproduce VSOR activity (Voss et al., 2014; Okada et al., 2017), that charge-modifying mutation of LRRC8A did not markedly alter the pore properties (Voss et al., 2014), and that VSOR-like activity produced by the reconstitution system lacked intracellular ATP dependence and inactivation kinetics (Syeda et al., 2016) fail to meet the above criteria 1, 4, and 5, respectively. Further investigation is thus required before the molecular identities of the main components of VSOR, which form the pore, are precisely determined.

Since the LRR motif is known to mediate protein-protein interactions (Kobe and Kajava, 2001), it is highly possible that LRRC8A can interact not only with other paralogs of the LRRC8 family but also with other proteins, which may include the VSOR pore-forming protein and/or its regulator protein. In fact, VSOR activity was drastically diminished when the 15 terminal LRRs of LRRC8A were truncated (Platt et al., 2017). In addition, the number of subunits in the LRRC8 heteromer containing LRRC8A and 8E was greater than six (Gaitán-Peñas et al., 2016). Furthermore, LRRC8A was shown to physically interact with TME-M16A/ANO1 and thereby regulate ANO1-mediated CaCC currents in *Xenopus* oocytes (Benedetto et al., 2016). In light of these facts, there remains a possibility that LRRC8A and/or its paralog(s) interacts with an additional or genuine VSOR pore-forming protein other than LRRC8 members. Since most of the LRR-containing protein families are known to share common functions such as an organizing role in intracellular Ca²⁺ signaling cascades (Abascal and Zardoya, 2012), it might be possible that LRRC8A somehow participates

Fig. 6. Effects of NOX expression on VSOR currents in HEK293T cells. (A) Representative VSOR whole-cell currents elicited by step pulses from -100 to $+100$ mV in 20 -mV increments during application of hypotonic (83% osmolality) solution in vector-transfected cells (upper panel) and NOX1-transfected cells (lower panel). (B) Mean VSOR currents recorded at $+100$ and -100 mV in vector-, NOX1-, NOX2-, NOX4-, and NOX5-transfected cells ($n = 5$ – 14). Experimental conditions are the same as reported previously (Okada et al., 2009a; Ando-Akatsuka et al., 2002).

in the Ca^{2+} -dependent type B mechanism for VSOR activation (Fig. 4).

Finally, it is noteworthy that a girl carrying a C-terminal truncated variant of LRRC8A, from whom the LRRC8 gene was first identified, was able to survive with only congenital agammaglobulinemia and minor facial anomalies (Sawada et al., 2003). Furthermore, LRRC8A knockout mice are not always embryonically lethal; some could be born alive, although they exhibit a variety of abnormalities (Kumar et al., 2014), in contrast to the indispensable roles of VSOR in cell survival and proliferation in all mammalian tissues. Moreover, clustered regularly interspaced short palindromic repeats-based screens showed that LRRC8A and 8C/8D/8E are not essential genes for proliferation and survival in human cancer cell lines (Wang et al., 2015). In addition, a recent study showed that swelling-induced VSOR-like currents were not suppressed, but rather were enhanced, by shRNA-mediated knockdown of LRRC8A in human retinal pigment epithelium (RPE) cells derived from induced pluripotent stem cells from a healthy donor (Milenkovic et al., 2015). These facts, on balance, may even suggest the possibility that LRRC8 members are necessary regulators for other VSOR pore-forming proteins. To elucidate whether the VSOR pore is formed by LRRC8 per se, researchers must verify that its charge-modifying mutant exhibits transition of the anion selectivity sequence from Eisenman type I to another Eisenman type or from anion selective to cation permeable or cation selective. Also, it must be pointed out that the channels reconstituted with the full set of core components should exhibit phenotypical properties including intracellular ATP requirements and intracellular free Mg^{2+} sensitivity.

To note, this article was submitted after the publication of several articles that show the hexameric structure of LRRC8A, mainly using cryo-electron microscopy

(Deneka et al., 2018; Kasuya et al., 2018; Kefauver et al., 2018), and suggest an involvement of LRRC8 in the VSOR pore using *LRRC8*^{-/-} HEK293 cells (Deneka et al., 2018) and HCT116 cells (Yamada and Strange, 2018; Zhou et al., 2018). Deneka et al. (2018) showed that the hexameric structure, which incorporates a pore-like, vase-shaped conformation, can be formed by purified recombinant LRRC8A alone or together with LRRC8C. In addition, the charge-neutralizing R103A mutant slightly increased the cation permeability of LRRC8A/8C heteromers, although the general preference of anions was still maintained, suggesting that the first extracellular loop is involved in the anion selectivity filter of VSOR (Deneka et al., 2018). However, at variance with this inference, a positive charge-conferring L105R mutant of LRRC8C failed to affect the anion selectivity of LRRC8A/8C heteromers. Yamada and Strange (2018) showed that a 25-amino-acid sequence unique to the intracellular loop of LRRC8A plays a role in VSOR pore structure and function. On the other hand, Zhou et al. (2018) demonstrated that the N termini of the LRRC8A/8C heteromer participate in forming the cytosolic portion of the VSOR pore. However, changes in the anion selectivity sequence and the anion/cation permeability ratio were not observed in these studies and are to be examined in future studies.

C. Maxi-Anion Channel

Anion channels with a single-channel conductance of 200 – 500 pS and with $P_{\text{Cl}}/P_{\text{Na}}$ or $P_{\text{Cl}}/P_{\text{K}} > 3$, called the MACs, have been observed in a wide variety of mammalian cell types (Sabirov et al., 2016), since the first observation in cultured muscle cells by Blatz and Magleby (1983). Thus far, we have observed MAC activity in mouse mammary C127 cells (Sabirov et al., 2001, 2017; Dutta et al., 2002; Sabirov and Okada,

2004b; Toychiev et al., 2009), rabbit macula densa cells (Bell et al., 2003), rat cardiomyocytes (Dutta et al., 2004, 2008), mouse astrocytes (Liu et al., 2006, 2008b), mouse and rat fibroblasts (Sabirov et al., 2006; Okada et al., 2009b; Toychiev et al., 2009), mouse thymocytes (Kurbannazarova et al., 2011), mouse L929 fibrosarcoma cells (Islam et al., 2012), and human U937 cells (Fig. 2C) under the same experimental conditions. We found that all of these cells share phenotypical properties, as follows: 1) single-channel conductance of 300–500 pS, 2) a linear (nonrectifying) current-voltage (I - V) relationship for instantaneous currents, 3) voltage-dependent inactivation kinetics at more positive and negative potentials than approximately ± 20 mV, and 4) high anion-to-cation selectivity without sensitivity to cation substitution or with $P_{\text{Cl}}/P_{\text{K}}$ or $P_{\text{Cl}}/P_{\text{Na}}$ of ≥ 6 . In addition, all of the large-conductance anion channels studied in our laboratory exhibited sharp sensitivity to a trivalent cation gadolinium (Gd^{3+}). Our previous critical analysis of all of the properties of large-conductance anion channels hitherto reported by our laboratory and others indicated that the channels reported in 61% of the original articles (51 of 84), including the first study (Blatz and Magleby, 1983), showed the above-described four phenotypical properties and those in 26% of the articles (22 of 84) were close to these properties (Sabirov et al., 2016). Thus, the MAC of this particular fingerprint was designated as “Maxi-Cl” (Sabirov et al., 2016).

Maxi-Cl is activated by osmotic swelling (Strange et al., 1996; Sabirov and Okada, 2009; Sabirov and Merzlyak, 2012; Sabirov et al., 2016) and therefore belongs to the VAAC group. As one of the VAACs, Maxi-Cl was expected to provide a Cl^- efflux pathway during the RVD process (Falke and Misler, 1989; Schlichter et al., 1990; Jalonen, 1993; Mitchell et al., 1997a) and the AVD process (Elinder et al., 2005). Maxi-Cl was also suggested to participate in the epithelial transport of Cl^- (McGill et al., 1993; Do et al., 2004) and HCO_3^- (Schneider et al., 1985; Becq et al., 1992; Riquelme et al., 2004) and in the charge balance in K^+ uptake in Schwann cells (McLarnon and Kim, 1991; Quasthoff et al., 1992). Our studies demonstrated that Maxi-Cl serves as a conductive pathway for the regulated release of ATP from a number of cell types (Sabirov et al., 2001, 2017; Dutta et al., 2002, 2004, 2008; Bell et al., 2003; Liu et al., 2008a,b; Islam et al., 2012) and glutamate from astrocytes (Liu et al., 2006), thereby playing a role in cell-to-cell signaling and being classified as a VSOAC.

1. Phenotypical Properties. First, Maxi-Cl is clearly distinguished from other anion channels by its very large unitary conductance (300–500 pS) under physiologic conditions. Multiple subconductance states have often been observed in the unitary events of Maxi-Cl (Bolotina et al., 1987; Bosma, 1989; Becq et al., 1992; Olesen and Bundgaard, 1992; Brown et al., 1993; Sun

et al., 1993; Dutta et al., 2004). The large single-channel conductance of Maxi-Cl implies its wide pore size. The fact that ATP anions with a mean diameter of 1.2–1.3 nm produce a fast open-channel block of Maxi-Cl (Sabirov et al., 2001) suggests that the diameter of its selectivity filter is close to or just slightly larger than 1.3 nm. Furthermore, permeability studies of Maxi-Cl to organic anions of different sizes in C127 cells suggested that the pore diameter at the narrowest portion is 1.1–1.5 nm (Sabirov and Okada, 2004b). In addition, our studies conducted using the nonelectrolyte partitioning method in C127 cells showed that Maxi-Cl appears to have a wider extracellular entrance with a diameter of approximately 2.8 nm and a narrower intracellular entrance with a diameter of 2.3 nm (Sabirov and Okada, 2004b).

The second phenotypical property of Maxi-Cl is its ohmic behavior, which distinguishes it from other VAACs (i.e., outwardly rectifying VSOR and inwardly rectifying ClC-2). The I - V relationship of the fully open state of unitary events is symmetrical and linear with no rectification (Fig. 2C). Even in macropatches, the voltage dependence of the instantaneous currents was linear in C127 cells (Sabirov et al., 2001) and rat cardiomyocytes (Okada et al., 2009b). However, the steady-state macropatch currents and whole-cell currents exhibited sigmoidal I - V relationships because of voltage-dependent inactivation.

The third phenotypic property of Maxi-Cl is its voltage- and time-dependent inactivation at more positive and negative potentials than approximately ± 20 mV (Fig. 2C). In other words, the channel stays open at 0 to ± 20 mV but exhibits voltage-dependent gating with rapid closing when the membrane potential exceeds approximately ± 20 mV. This property reflects the bell-shaped dependence of Maxi-Cl open channel probability, with the maximum at a certain voltage near 0 mV in a variety of cell types (Bosma, 1989; Hals et al., 1989; Jalonen et al., 1989; Schlichter et al., 1990; McGill et al., 1992; Pahapill and Schlichter, 1992; Sun et al., 1992, 1993; Bettendorff et al., 1993; Brown et al., 1993; Forshaw et al., 1993; Jalonen, 1993; Hardy and Valverde, 1994; Mitchell et al., 1997a; Riquelme and Parra, 1999; O'Donnell et al., 2001; Sabirov et al., 2001; Do et al., 2004; Riquelme et al., 2004; Elinder et al., 2005; Akanda and Elinder, 2006).

The fourth phenotypic property of Maxi-Cl is its strong anion selectivity. The I - V relationship and the reversal potential of Maxi-Cl currents are often unaffected by substitution of a large cation such as N -methyl-D-glucamine $^+$ or TEA $^+$ for Na^+ or K^+ in bathing solution. Even if Maxi-Cl exhibited very limited cation permeability, the channel well discriminates anions from cations with a very high permeability ratio of chloride over sodium or potassium ($P_{\text{Cl}}/P_{\text{Na}}$ or $P_{\text{Cl}}/P_{\text{K}}$) of 20–45 (Coulombe et al., 1987; Falke and Misler, 1989; Hals et al., 1989; McCann et al., 1989;

Schlichter et al., 1990; Coulombe and Coraboeuf, 1992; Pahapill and Schlichter, 1992; Bettendorff et al., 1993; Mitchell et al., 1997a; Sabirov et al., 2001) or a fairly high $P_{\text{Cl}}/P_{\text{Na}}$ or $P_{\text{Cl}}/P_{\text{K}}$ value of 6–18 (Light et al., 1990; McGill et al., 1992; Sun et al., 1992, 1993; Bajnath et al., 1993; Riquelme et al., 1995; O'Donnell et al., 2001; Do et al., 2004; De Marchi et al., 2008). Maxi-Cl exhibits weak field anion selectivity with an Eisenman type I permeability sequence of $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^-$ (Schneider et al., 1985; Soejima and Kokubun, 1988; Bosma, 1989; Schlichter et al., 1990; Kokubun et al., 1991; Pahapill and Schlichter, 1992; Bajnath et al., 1993; Hardy and Valverde, 1994; Sabirov et al., 2001; Sabirov and Okada, 2004b; Toychiev et al., 2009).

2. Activation Mechanisms. Maxi-Cl is normally silent or inactivated but becomes activated by a variety of physiologic or pathophysiological stimuli. Its on-cell mode activation is induced by hypo-osmotic stimulation (Falke and Mislér, 1989; Coulombe and Coraboeuf, 1992; Jalonen, 1993; Sabirov et al., 2001; Dutta et al., 2002, 2004, 2008; Liu et al., 2006; Kurbannazarova et al., 2011), salt stress (Bell et al., 2003), G protein-coupled receptor stimulation (Kawahara and Takuwa, 1991; Sun et al., 1992, 1993), and ischemic or hypoxic stimulation (Dutta et al., 2004; Liu et al., 2006, 2008a). Patch membrane excision is an effective stimulus to activate Maxi-Cl (Bosma, 1989; Kanno and Takishima, 1990; Light et al., 1990; Schlichter et al., 1990; Kawahara and Takuwa, 1991; Coulombe and Coraboeuf, 1992; Bajnath et al., 1993; Jalonen, 1993; Mitchell et al., 1997a; Sabirov et al., 2001, 2006; Dutta et al., 2002, 2004; Bell et al., 2003; Liu et al., 2006; Toychiev et al., 2009; Kurbannazarova et al., 2011; Islam et al., 2012).

In the cell-free excised mode, Maxi-Cl activity is independent of intracellular Ca^{2+} in the physiologic concentration range and is not largely affected by intracellular Ca^{2+} chelation (Blatz and Magleby, 1985; Soejima and Kokubun, 1988; Bosma, 1989; Falke and Mislér, 1989; McCann et al., 1989; Light et al., 1990; Becq et al., 1992; Coulombe and Coraboeuf, 1992; Olesen and Bundgaard, 1992; Pahapill and Schlichter, 1992; Sun et al., 1992; Bell et al., 2003; Toychiev et al., 2009), whereas its on-cell activity is induced by the application of a Ca^{2+} ionophore, A23187 [5-(methylamino)-2-((2R,3R,6S,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl)methyl)-1,3-benzoxazole-4-carboxylic acid] (Light et al., 1990; Kawahara and Takuwa, 1991; Bajnath et al., 1993; Sun et al., 1993).

Maxi-Cl activity in excised patches was activated even in the absence of cytosolic ATP (Soejima and Kokubun, 1988; McGill et al., 1992; Olesen and Bundgaard, 1992). In contrast, the unitary conductance of Maxi-Cl was reduced by 5 mM cytosolic ATP (Bettendorff et al., 1993), and the mean number of

Maxi-Cl open channels was markedly suppressed by cytosolic Mg-ATP in a concentration-dependent manner with an IC_{50} of 29.2 μM (Toychiev et al., 2009). A broad-spectrum serine/threonine protein kinase inhibitor, 5-(2-methylpiperazin-1-yl)sulfonylisoquinoline dihydrochloride (H7), activated Maxi-Cl-like activity in vascular smooth muscle A7r5 cells (Saigusa and Kokubun, 1988) but had no effect on Maxi-Cl activity in mammary C127 cells (Toychiev et al., 2009). In contrast, protein kinase C was reported to activate a non-Maxi-Cl type of MAC in renal cortical collecting duct RCCT-28 cells (Schwiebert et al., 1992). Another non-Maxi-Cl type of MAC in mouse neuroblastoma C1300 cells was inhibited by a serine/threonine protein phosphatase inhibitor, okadaic acid (Díaz et al., 2001). In contrast, Maxi-Cl activity in C127 cells subjected to hypotonic stimulation was insensitive to okadaic acid but was largely inhibited by a number of broad-spectrum protein tyrosine phosphatase inhibitors (Toychiev et al., 2009). Furthermore, even without hypotonic stimulation, protein tyrosine kinase (PTK) inhibitors brought the channel out of an inactivated state in C127 cells and mouse adult skin fibroblasts (Toychiev et al., 2009) as well as in L929 cells (Islam et al., 2012). A receptor protein tyrosine phosphatase ζ was identified to be, at least in part, responsible for activation of Maxi-Cl in mouse adult skin fibroblasts (Toychiev et al., 2009). Taken together, it appears that activation of Maxi-Cl involves protein dephosphorylation mediated by protein tyrosine phosphatases.

3. Molecular Identity. Despite a long history of research on Maxi-Cl, including its well-defined phenotypic properties and important functions, the channel's molecular nature remained undetermined until quite recently. At least four candidates had been proposed for the Maxi-Cl protein, including the plasmalemmal subtype of mitochondrial voltage-dependent anion channel, ADP/ATP carrier AAC, *twisty* TTYH1, and connexin 43, but all were disproved (for reviews, see Sabirov and Okada, 2005, 2009; Okada et al., 2009b, 2018). Thus, we conducted further studies by applying unbiased genome-wide approaches (Islam et al., 2013). By adopting the strategy as summarized in Fig. 7, we recently determined the molecular identity of the Maxi-Cl core component (called MAC-1) as solute carrier organic anion transporter family member SLCO2A1 (Sabirov et al., 2017). Our starting materials were membrane blebs isolated from the mouse mammary C127 cell line, which exhibits the highest activity of Maxi-Cl studied thus far (Sabirov et al., 2001). Functional Maxi-Cl was found to be accumulated in the constitutively activated state in the blebs. After solubilization of the total bleb-membrane proteins, we selected a fraction with the highest Maxi-Cl activity and performed a proteomics analysis by subjecting the fraction to liquid chromatography-tandem mass spectrometry, yielding 439 genes. Among them, we picked up 15 genes

Fig. 7. Strategy adopted in our study for molecular identification of the core component of Maxi-Cl. By performing bleb membrane proteomics followed by gene silencing and disruption, mutagenesis, pharmacology, heterologous expression, and recombinant protein reconstitution, *SLCO2A1* protein was identified as a core component of the ATP-conductive Maxi-Cl channel (see the text and Sabirov et al., 2017 for details). LC, liquid chromatography; MS/MS, tandem mass spectrometry.

that encode proteins with multiple transmembrane-spanning domains and observed the effects of their gene silencing on Maxi-Cl activity. Only siRNA- and miRNA-mediated knockdown of the *SLCO2A1* gene was reproducibly effective in suppressing Maxi-Cl activity. Since *SLCO2A1* is known as the molecule of prostaglandin transporter (PGT), we tested the effects of PGT substrate PGE2 and PGT blockers bromosulphophthalein (BSP), bromocresol green, and indocyanine green; we found that all of these compounds could suppress Maxi-Cl currents. When *SLCO2A1* was heterologously expressed in HEK293T cells, which do not endogenously express *SLCO2A1* and thus lack Maxi-Cl activity, BSP-sensitive Maxi-Cl activity emerged. Mutants of *SLCO2A1*, G222R and P219L, which are associated with pachydermoperiostosis (Zhang et al., 2012, 2014), never emerged the channel activity, despite their successful plasmalemmal expression. On the other hand, over-expression of the charge-neutralized K613G mutant, which is known to impair *SLCO2A1* transporter function (Chan et al., 2002), produced channel activity with markedly reduced single-channel conductance that was relatively more selective to cations. These data suggest that *SLCO2A1* is involved in the pore formation of Maxi-Cl. To confirm this, we then reconstituted purified recombinant *SLCO2A1* proteins into lipid bilayers. Reconstituted *SLCO2A1* exhibited Maxi-Cl activity that was sensitive to Gd³⁺ and PGE2. The charge-neutralized K613G mutant was again more permeable to cations,

exhibiting a smaller single-channel conductance. Taken together, we conclude that *SLCO2A1* constitutes the core component or the pore of Maxi-Cl.

In light of the above results, it is deemed that *SLCO2A1* functions in two modes: 1) as a PGT in the resting state presumably with phosphorylated tyrosine residue(s) and 2) as an ATP-conductive Maxi-Cl channel in the activated state with dephosphorylated tyrosine residue(s); in addition, the transition from the resting to activated state is induced by membrane excision, cell swelling, ischemia, or hypoxia, as schematically depicted in Fig. 8. It is striking that the loss of a multigate barrier in a transporter often leads to anion channel activity, as pointed out by Minor (2017). Such bimodal channel/transporter proteins include ClC family members functioning as Cl⁻ channels and Cl⁻/H⁺ exchangers (Picollo and Pusch, 2005; Scheel et al., 2005; Accardi and Picollo, 2010), SLC1As serving as excitatory amino acid transporters and anion channels (Cater et al., 2016; Fahlke et al., 2016), SLC26A3/6 serving as Cl⁻ channels and anion exchangers (Ohana et al., 2009), and TMEM16F and fungal TMEM16 serving as CaCCs and lipid scramblases (Malvezzi et al., 2013; Suzuki et al., 2014; Picollo et al., 2015).

Maxi-Cl in membrane blebs derived from C127 cells as well as the channel reconstituted from proteins isolated from the blebs and recombinant *SLCO2A1* proteins into the proteoliposomes were all in a constitutive active state (Sabirov et al., 2017), suggesting that

Fig. 8. Dual modes of SLCO2A1 functions as a transporter, PGT (left), and an ATP-permeable maxi-anion channel, Maxi-Cl (right).

the whole Maxi-Cl complex natively contains or is associated with some regulatory component(s) for channel activation. In addition, Maxi-Cl activity reconstituted with recombinant SLCO2A1 proteins was totally insensitive to BSP (Sabirov et al., 2017), indicating that some BSP-sensitive component is missing in the reconstituted channel. Therefore, it appears that some additional component(s) other than the core component SLCO2A1 is required for the whole Maxi-Cl complex. We must also point out that there should be some as-yet-unidentified molecules other than SLCO2A1 responsible for non-Maxi-Cl-type MACs other than MAC-1. Those missing components for Maxi-Cl and non-Maxi-Cl MAC molecules are to be identified in future studies.

III. Volume-Correlated Anion Channels

As noted in section I, three types of VCACs (CFTR, CaCC, and ASOR) are activated by cAMP, Ca^{2+} , and acidification, respectively, but not by cell volume changes. Hence, they cannot be professionally involved in cell volume regulation. Nevertheless, in some situations, they are implicated in cell volume changes.

A. Cystic Fibrosis Transmembrane Conductance Regulator

Disorder of the CFTR gene is responsible for monogenic cystic fibrosis in humans (Riordan et al., 1989). CFTR is a unique member of the ABC transporter superfamily, which functions as a low-conductance anion channel rather than as a transporter, and is expressed principally in epithelial tissues and also in some nonepithelial tissues, including cardiomyocytes and smooth muscle cells (Anderson et al., 1991; Kartner et al., 1991; for review, see Hwang and Sheppard, 2009).

CFTR is phenotypically characterized by biophysical properties exhibiting low single-channel conductance (6–10 pS), a linear I - V relationship, high-field anion

selectivity ($\text{Br}^- > \text{Cl}^- > \text{I}^-$: Eisenman type III selectivity sequence), and voltage independence without exhibiting any voltage-dependent activation and/or inactivation kinetics (Sheppard and Welsh, 1999). Phosphorylation of the intracellular regulatory domain (RD) mainly attained by PKA is a prerequisite to CFTR channel opening (Hegedus et al., 2009). Recent studies using electron cryomicroscopy revealed that phosphorylation of RD becomes disengaged from its inhibitory position docked inside the intracellular vestibule (Liu et al., 2017; Zhang et al., 2017). Transitions between open and closed states of CFTR channels are directly induced by ATP binding and hydrolysis on the cytosolic nucleotide binding domains (NBD1 and NBD2), which can form a “head-to-tail” dimer (Kirk and Wang, 2011). CFTR was recently reported to be directly (in a manner independent of PKA) activated by membrane stretch (Zhang et al., 2010a) and osmotic cell swelling (Xie et al., 2016). The conclusions deduced in both studies were mainly based on pharmacological observations using CFTR_{inh}-172 (3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone) and GlyH-101 [N-(2-naphthalenyl)-((3,5-dibromo-2,4-dihydroxyphenyl)methylene)glycine hydrazide] as CFTR-specific antagonists, although these drugs were known to also block VSOR (Melis et al., 2014; Friard et al., 2017) and ClC-2 (Cuppoletti et al., 2014). Moreover, these reports did not provide enough biophysical evidence to convince the readers that recorded channel events exhibit the phenotypic characteristics of CFTR. Thus, at present, CFTR cannot be classified as a VAAC.

CFTR is a multifunctional protein that functions not only as an anion channel but also as a regulator for a number of other ion channels (Kunzelmann and Schreiber, 1999; Schwiebert et al., 1999). For instance, CFTR regulates the epithelial amiloride-sensitive Na^+ channel ENaC (Kunzelmann et al., 1997; Schreiber et al., 1999), renal outer medullary K^+ channel 2 (McNicholas et al., 1997), and the outwardly rectifying

(depolarization-induced) Cl^- channel (Fulmer et al., 1995; Jovov et al., 1995) through protein-protein interactions mediated by NBD1, as well as CaCC mediated by RD (Wei et al., 2001) and VSOR mediated by NBD2 (Ando-Akatsuka et al., 2002).

Activation of CFTR in epithelial cells causes Cl^- secretion and thus often leads to cell shrinkage, called a secretory volume decrease (Manabe et al., 2004). For example, daidzein-induced activation of CFTR resulted in shrinkage of renal epithelial cells due to a decrease in $[\text{Cl}^-]_i$ (Marunaka, 1997). However, genistein-induced CFTR activation did not result in cell shrinkage, because simultaneous activation of NKCC maintained the $[\text{Cl}^-]_i$ level (Niisato et al., 1999) by compensating for CFTR-mediated Cl^- efflux with NKCC-mediated Cl^- influx (see Fig. 1).

A splice variant of CFTR is expressed in a variety of mammalian cardiomyocytes (Levesque et al., 1992; Hart et al., 1996; James et al., 1996) and plays roles in regulating action potential duration and establishing resting potential (McCarty, 2000). Cardiac CFTR was found to be involved in the RVD process under particular conditions in which β -adrenergic receptors were stimulated and the intracellular cAMP level was thereby increased (Wang et al., 1997; Yamamoto et al., 2001).

Cell volume regulation after a hypotonic challenge (RVD) was defective in intestinal crypt epithelial cells isolated from CFTR-knockout mice, and this was caused by the inability of K^+ conductance but not by CFTR Cl^- conductance (Valverde et al., 1995). In fact, a CF mutation (ΔF508) or knockdown of CFTR impaired RVD owing to a lack of swelling-induced activation of intermediate-conductance Ca^{2+} -dependent K^+ channel IK1, presumably through an interaction between CFTR and potassium calcium-activated channel KCNN4 (Vázquez et al., 2001). In addition, CFTR expression was reported to facilitate the RVD process by augmenting swelling-induced ATP release mediated by the Gd^{3+} -sensitive non-CFTR anion channel (Hazama et al., 2000; Braunstein et al., 2001). In an autocrine fashion, released ATP may stimulate purinergic P2 receptors and then induce an increase in intracellular Ca^{2+} , thereby facilitating RVD due to activation of both Ca^{2+} -dependent K^+ channels (Dezaki et al., 2000) and VSORs (Akita et al., 2011). In fact, CFTR knockout was found to abolish swelling-activated, Ca^{2+} -activated K^+ channel currents (Belfodil et al., 2003) as well as VSOR currents and RVD (Barrière et al., 2003) in mouse renal tubular cells. Taken together, we conclude that noncardiac CFTR is indirectly involved in RVD efficacy.

CFTR permeates organic anions including GSH (Linsdell and Hanrahan, 1998; Kogan et al., 2003), but not ATP (Linsdell and Hanrahan, 1998), and actually mediates release of GSH in the lung (Kariya et al., 2007) and renal tubular cells (l'Hoste et al., 2010).

Phosphorylation-dependent dilation of the pore lumen with the radii of the external vestibule (0.92 nm) and the narrowest part (0.69 nm) may account for this function (Krasilnikov et al., 2011). Thus, CFTR can also be classified as a VSOAC.

B. Calcium-Activated Chloride Channel

CaCC activity was discovered in *Xenopus* oocytes (Miledi, 1982) and salamander photoreceptors (Bader et al., 1982). CaCC, which shows anion selectivity with an Eisenman type I sequence, was subsequently shown to be expressed in a wide variety of mammalian tissues playing fundamental roles in physiologic processes such as transepithelial transport of electrolytes and fluid, regulation of smooth muscle contractility, modulation of cardiac and neuronal action potentials, control of signaling in photoreceptors and olfactory receptors, and cancer cell proliferation (Hartzell et al., 2005; Huang et al., 2012a). CaCC opens in response to increases in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The single-channel conductance of CaCC is approximately 8 pS (Nilius et al., 1997b; Piper and Large, 2003; Crutzen et al., 2016). CaCC currents exhibit outward rectification and voltage dependence and display activation and deactivation kinetics upon depolarization and hyperpolarization, respectively, in the physiologic range of $[\text{Ca}^{2+}]_i$, whereas the currents become time and voltage independent and ohmic (I - V linear) at higher $[\text{Ca}^{2+}]_i$ (Frings et al., 2000; Kuruma and Hartzell, 2000).

The first candidate proposed for the molecular identity of CaCC was CLCA1, the cDNA of which was isolated in 1995 from a bovine tracheal cDNA library (Cunningham et al., 1995). However, the biophysical fingerprint of CLCA1, which lacks voltage-dependent kinetics and outward rectification and exhibits sensitivity to reducing agents such as dithiothreitol, is quite different from that of CaCC, and there is no correlation of endogenous expression between CaCC activity and CLCA1 mRNA (Eggermont, 2004). Thus, CLCA1 is distinct from the molecule responsible for CaCC, although there remains a possibility that CLCA1 is an accessory component for the CaCC complex or an activating regulator for CaCC (Loewen and Forsyth, 2005; Sala-Rabanal et al., 2015).

Another family, bestrophin (Best), was then proposed to confer CaCC. Human Best1 (hBest1), which was identified as the gene responsible for Best vitelliform macular dystrophy (also called Best's disease) (Marquardt et al., 1998; Petrukhin et al., 1998), was shown to function as a CaCC molecule (Sun et al., 2002). Actually, many of the disease-causing mutations in hBest1 were found to cause dysfunction of CaCC (Xiao et al., 2010). CaCC activity was subsequently shown to be associated with Best1 of *Drosophila* (Chien et al., 2006) and mice (mBest1; Park et al., 2009). In addition, similar CaCC activity was reported for Best2 (Qu et al., 2003b; Pifferi et al., 2006), Best3

(Matchkov et al., 2008; O'Driscoll et al., 2008), and Best4 (Tsunenari et al., 2006). However, some properties of Best-associated Ca^{2+} -activated Cl^- currents were distinct from the classic CaCC phenotype. In particular, Best-associated currents did not show clear outward rectification and voltage-dependent activation and inactivation kinetics (Qu et al., 2003b; Chien et al., 2006; Pifferi et al., 2006; O'Driscoll et al., 2008). Moreover, endogenous CaCC currents were normally observed in RPE cells isolated from mBest1 knockout mice (Marmorstein et al., 2006) and salivary acinar cells derived from mBest2 knockout mice (Romanenko et al., 2010). Also, RPE cells derived from knock-in mice carrying the Best vitelliform macular dystrophy-causing mutant (W93C) of mBest1 exhibited normal CaCC conductances (Zhang et al., 2010b). Furthermore, Best1 was recently shown to be a multifunctional protein serving as a regulator for the voltage-gated Ca^{2+} channel (Marmorstein et al., 2006; Rosenthal et al., 2006; Yu et al., 2008) and for Ca^{2+} release from the ER (Barro-Soria et al., 2010; Neussert et al., 2010; Zhang et al., 2010b; Gómez et al., 2013). On the other hand, not only *Drosophila* Best1 but also hBest1 and mBest2 are shown to be sensitive to changes in cell volume (Fischmeister and Hartzell, 2005; Chien and Hartzell, 2007; Xiao et al., 2009), although it remains unclear how cell volume changes are sensed by Best.

Finally, TMEM16A/ANO1 has been shown independently by three groups to exhibit a full set of the phenotypic properties of CaCC and to be the molecular identity of CaCC (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). The TMEM16/ANO family has 10 members, all with 10 transmembrane domains, and plays a variety of physiologic roles in mammalian cells (Pedemonte and Galletta, 2014; Oh and Jung, 2016). TMEM16A/ANO1 shows approximately 60% sequence identity with TMEM16B/ANO2 but only 20%–30% sequence identity with remaining TMEM16C–K/ANO3–10. In fact, TMEM16B/ANO2 is also shown to be a CaCC in photoreceptors (Stöhr et al., 2009), olfactory sensory neurons (Pifferi et al., 2009; Stephan et al., 2009; Billig et al., 2011), and hippocampal neurons (Huang et al., 2012b). On the other hand, TMEM16A/ANO1 and TMEM16B/ANO2 were recently found to be coexpressed, exhibit molecular interaction with each other, and thereby form heteromeric channels generating CaCC currents in rat pineal glands (Yamamura et al., 2018). Although TMEM16C–G/ANO3–7 are shown to be intracellular proteins (Duran et al., 2012), TMEM16F/ANO6 exhibited CaCC activity, when overexpressed, on the plasma membrane and exhibited “noncanonical” characteristics such as lower sensitivity to intracellular Ca^{2+} increases (Grubb et al., 2013; Shimizu et al., 2013). On the other hand, under certain conditions, TMEM16F/ANO6, the defective mutation of which is associated with the congenital bleeding disorder Scott syndrome in humans (Suzuki et al., 2010), served as a Ca^{2+} -dependent

scramblase (Suzuki et al., 2010) and as a Ca^{2+} -activated nonselective cation channel (Yang et al., 2012; Adomaviciene et al., 2013; Yu et al., 2015).

CaCC activation is often associated with cell volume changes. For example, muscarinic stimulation of intestinal epithelial cells with carbamylcholine induced fluid secretion and cell shrinkage or secretory volume decrease (Valverde et al., 1993; Manabe et al., 2004) due to CaCC-mediated Cl^- secretion. Stimulation with Ca^{2+} ionophore A23187 and neuropeptide bradykinin is known to result in swelling in platelets and glioma cells, respectively, due to Cl^- influx mediated by CaCC activation (Fine et al., 1994; Seifert and Sontheimer, 2014). In addition, CaCC activity was also reported to be indirectly involved in the RVD process in low intracellular Ca^{2+} buffering conditions, because siRNA-mediated knockdown of TMEM16A/ANO1 and of TMEM16F/ANO6 slowed but did not abolish the RVD process in HEK293 and Ehrlich ascites tumor cells, respectively (Almaça et al., 2009; Juul et al., 2014). RVD was also still observed, albeit slowly, in the absence of extracellular Ca^{2+} , although the RVD-facilitating effect of CaCC mediated by TMEM16F/ANO6 was abolished under the Ca^{2+} -free condition (Juul et al., 2014). Thus, it is possible that volume-regulatory Cl^- efflux is mediated, in part, by CaCC. In addition, the time course of RVD was recently found to be accelerated in TMEM16F/ANO6-overexpressing HEK293 cells and slowed (but was not abolished) in intestinal epithelial cells isolated from TMEM16F/ANO6 knockout mice (Sirianant et al., 2016). Thus, it is also possible that TMEM16F/ANO6 acts as a Ca^{2+} -activated nonselective cation channel and serves as a pathway for Ca^{2+} entry, which is required for volume-regulatory operation of Ca^{2+} -activated K^+ channels.

C. Acid-Sensitive Outwardly Rectifying Anion Channel

ASOR, which is rapidly activated by extracellular acidification (at $\text{pH} < 6$), is expressed in a variety of mammalian cell types, including human epithelial HEK293 or HEK293T cells (Nobles et al., 2004; Lambert and Oberwinkler, 2005; Matsuda et al., 2010a; Okada et al., 2014; Capurro et al., 2015), HeLa cells (Wang et al., 2007; Sato-Numata et al., 2013, 2016, 2017), human red blood cells (Kucherenko et al., 2009), human monocytic leukemia THP-1 cells (Fu et al., 2013), human bronchial epithelial cell lines (Capurro et al., 2015), pancreatic adenocarcinoma CFPAC cells (Capurro et al., 2015), neuroblastoma SK-N-MC cells (Capurro et al., 2015), mouse hippocampal astrocytes (Lambert and Oberwinkler, 2005), mouse smooth muscle cells (Matsuda et al., 2010a), mouse cortical neurons (Sato-Numata et al., 2014), mouse RAW264.7 macrophages (Capurro et al., 2015), mouse and guinea pig cardiac myocytes (Yamamoto and Ehara, 2006), rat Sertoli cells (Auzanneau et al., 2003), and Fischer rat thyroid (FRT) cells (Capurro et al., 2015). This channel

may be activated and play some physiologic or patho-physiologic roles under strong acidic conditions, such as in the inner medullary collecting duct, where the acidity of tubular filtrate can decrease below pH 5.5 (Bengele et al., 1983, 1986), and in the brain subjected to ischemia, seizure, trauma, or hyperglycemia (Kraig et al., 1985; Rehncrona, 1985; Siesjö, 1988; Chesler and Kaila, 1992).

Whole-cell ASOR current is phenotypically characterized by acid sensitivity, strong outward rectification, and activation kinetics upon application of positive voltages (Nobles et al., 2004; Lambert and Oberwinkler, 2005; Wang et al., 2007; Sato-Numata et al., 2014; Capurro et al., 2015). ASOR current was recently found to be highly sensitive to temperature, with Q_{10} values of 8.8 in human epithelial HeLa cells and 5.6 in mouse cortical neurons (Sato-Numata et al., 2013, 2014). In the physiologic range of membrane potential, the single-channel conductance of ASOR is 4–7 pS in HEK293 cells (Lambert and Oberwinkler, 2005) and 4.8 pS in HeLa cells (Wang et al., 2007). Previous studies showed that regulatory mechanisms of ASOR activation do not involve intracellular Ca^{2+} , ATP, and G proteins (Yamamoto and Ehara, 2006) or protein kinases A and C (Capurro et al., 2015). Pharmacological studies suggested that ASOR is regulated by mitochondrion-generated ROS (Fu et al., 2013) as well as PTK and phosphatidylinositol-3-kinase (Capurro et al., 2015). In pharmacological studies, ASOR regulation was suggested to be dependent on exocytosis/endocytosis (Capurro et al., 2015), which contradicts the independence of intracellular Ca^{2+} and ATP.

The molecular identity of ASOR is yet to be determined. Since extracellular acidification was observed to activate anionic currents conducted via a number of ClC channel/transporter family members, including ClC-0 (Chen and Chen, 2001), ClC-2 (Jordt and Jentsch, 1997; Stroffekova et al., 1998), ClC-3 (Matsuda et al., 2010a), and ClC-7 (Diewald et al., 2002; Kajiya et al., 2009), it is possible that some ClC members are associated with the molecular entity of ASOR. ClC-3 (Matsuda et al., 2010a; Wang et al., 2012) and ClC-7 (Kajiya et al., 2009; Ohgi et al., 2011) were suggested to be responsible for the ASOR molecule. However, this possibility was recently ruled out in light of the following observations. First, both ClC-3 and ClC-7 were localized in the intracellular compartments but not on the plasma membrane (Auzanneau et al., 2003). Second, endogenous ASOR currents were not affected by siRNA-mediated knockdown of ClC-3 (Sato-Numata et al., 2013; Capurro et al., 2015) and ClC-7 (Wang et al., 2012; Capurro et al., 2015). Third, ASOR currents were not enhanced in cells overexpressing ClC-3a (Sato-Numata et al., 2013), ClC-3d (Okada et al., 2014), and ClC-7 (Sato et al., 2012).

The sharp outward rectification, voltage-dependent activation kinetics, and temperature sensitivity of ASOR currents are similar to those of TMEM16A/ANO1 currents. Thus, it is possible that some member of TMEM16/ANO is associated with the ASOR molecule. Inconsistently, however, ASOR currents were found to be insensitive to siRNA-mediated knockdown of not only TMEM16A but also TMEM16D, F, H, and K (Capurro et al., 2015).

Based on similarities in some pharmacological properties and the peak amplitudes between ASOR and VSOR currents, it was previously assumed that both anion channels share the same molecular entity with two different manifestations being exhibited (Nobles et al., 2004). However, there is a distinct difference in the time lag of activation after stimulation between immediately activating ASOR and slowly activating VSOR currents. As noted in section II.B, phenotypical properties of VSOR are distinct from those of ASOR, which include cytosolic ATP independence (Yamamoto and Ehara, 2006), cytosolic Mg^{2+} insensitivity (Lambert and Oberwinkler, 2005), a one-digit smaller single-channel conductance (Wang et al., 2007), and much steeper outward rectification (Auzanneau et al., 2003; Nobles et al., 2004; Lambert and Oberwinkler, 2005; Wang et al., 2007). In addition, ASOR currents are totally insensitive to a number of putative VSOR blockers, as discussed in section IV. Furthermore, both VSOR and ASOR currents were found to be simultaneously activated in an additive manner (Lambert and Oberwinkler, 2005; Yamamoto and Ehara, 2006; Matsuda et al., 2010b). Taken together, it is now clear that the molecular identity of ASOR is distinct from that of VSOR. In this regard, it is noteworthy that ASOR currents were not affected by siRNA-mediated knockdown of either LRRC8A, which is an essential component of VSOR (see section II.B), or LRRC8B/C/D/E (Sato-Numata et al., 2016).

Activation of ASOR was associated with persistent cell swelling, called necrotic volume increase (NVI), which is followed by necrotic cell death in human epithelial HeLa cells (Wang et al., 2007) and mouse cortical neurons (Sato-Numata et al., 2014) under acidotoxic conditions. The NVI process is known to be induced by water inflow driven mainly by NaCl influx (Barros et al., 2001; Okada et al., 2001, 2004). To obtain NaCl influx, some Na^+ -permeable cation channels must be activated when anionic ASOR currents are activated, according to electroneutrality principle, thereby giving rise to depolarization, which in turn drives ASOR-mediated Cl^- inflow. In HeLa cells, TRPM7 was identified to represent this cation channel activated by reduced blocking effects of extracellular divalent cations under acidotoxic conditions (Numata and Okada, 2009). In neuronal cells under acidotoxic conditions, the voltage-insensitive, acid-sensitive Na^+ -permeable cation acid-sensing ion channel

may represent this cationic conduction pathway to attain NVI (Xiong et al., 2004; Yermolaieva et al., 2004). Thus, ASOR is involved in cell volume changes in acidic situations in which some cation channels are simultaneously activated.

IV. Pharmacological Properties of and Distinctions between Volume-Activated Anion Channels and Volume-Correlated Anion Channels

Pharmacological information on anion channels is important for the investigation of their properties, activation mechanisms, and functions and ultimately the development of drugs for clinical uses. In general, most anion channel blockers exhibit broad specificity that prevents sharp discrimination of one type of anion channel from the others. Since the molecular entities of most anion channels or their core components have been identified, accumulating pieces of information on the basis of molecular understanding have progressively been added to anion channel pharmacology.

A. ClC-2-Type Chloride Channel Blockers

A group 12 heavy metal ion, Cd^{2+} , which is a common air pollutant and a major component of cigarette smoke, is the most effective and well established blocker of ClC-2 (for transfected ClC-2, see Schwiebert et al., 1998; Zúñiga et al., 2004; Cuppoletti et al., 2013; for endogenous ClC-2, see Chesnoy-Marchais and Fritsch, 1994; Bond et al., 1998; Clark et al., 1998; Enz et al., 1999; Blaisdell et al., 2000; Duan et al., 2000) with an IC_{50} of 48 μM (Zúñiga et al., 2004) (see Table 2). Cd^{2+} is also known to block ClC-1 channel activity (Fahlke et al., 1998) and Cl^- channel currents mediated by ClC-3a and ClC-3d overexpressed in the plasma membrane (Okada et al., 2014). Cd^{2+} is ineffective for VSOR (Fritsch and Edelman, 1997; Bond et al., 1998; Kajita et al., 2000;

Parkerson and Sontheimer, 2004) and CFTR (Wang et al., 2009; Infield et al., 2016) as well as for Maxi-Cl (see Fig. 12A) and ASOR (see Fig. 12B), whereas Cd^{2+} sensitivity of CaCC has not been studied thus far. In addition, it must be noted that voltage-gated Ca^{2+} channels are also blocked by Cd^{2+} (Hagiwara and Byerly, 1981; Lansman et al., 1986). Cd^{2+} does not affect activation but greatly accelerates the deactivation gate by binding to cysteine residue C256 in ClC-2 (Zúñiga et al., 2004). Another group 12 metal, Zn^{2+} , blocks ClC-2 (Clark et al., 1998; Schwiebert et al., 1998; Huber et al., 2004) with an IC_{50} of 23 μM (Clark et al., 1998). However, it is noted that Zn^{2+} blocks not only ClC-2 but also ClC-1 (Kürz et al., 1997, 1999) and Maxi-Cl (Schlichter et al., 1990; Kokubun et al., 1991; O'Donnell et al., 2001).

A carboxylate analog, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), partially and voltage-dependently blocks ClC-2 (Clark et al., 1998; Furukawa et al., 1998; Xiong et al., 1999; Thompson et al., 2005) with a low affinity ($\text{IC}_{50} = 0.98 \text{ mM}$; Furukawa et al., 1998). However, its specificity with inhibiting VSOR, Maxi-Cl, CFTR, and CaCC is very low, although NPPB was totally ineffective in blocking ASOR (Sato-Numata et al., 2016). ClC-2 is partially sensitive to other carboxylate analogs, anthracene-9-carboxylic acid (9-AC) and diphenylamine-2-carboxylate (DPC) (Thiemann et al., 1992; Clark et al., 1998; Schwiebert et al., 1998).

A scorpion venom peptide toxin, GaTx2, is a high-affinity ClC-2 blocker ($\text{IC}_{50} = 20 \text{ pM}$) that is selective over other ClC family members (ClC-0, ClC-1, ClC-3, and ClC-4), CFTR, CaCC, and the GABA_C receptor (Thompson et al., 2009). GaTx2 inhibits ClC-2 in a manner independent of voltage by slowing the activation gate without affecting deactivation and open channel conductance (Thompson et al., 2009). A synthetic opiate, methadone, was reported to voltage-independently block ClC-2 with an IC_{50} of 100 nM

TABLE 2
Pharmacological distinctions among VAACs and VCACs

Chemical	ClC-2	VSOR	Maxi-Cl	CFTR	CaCC	ASOR
Cd^{2+}	+	—	—	—	NS	—
Gd^{3+}	—	—/+ ^a	+~+++	—	—	—
DCPIB	—	++	—	—	—	—
DIDS (out)	—	±~++ VD	+	—	±~+ VD	++~+++
NPPB	± VD	+	+	+++ VD	+	—
NFA	—	±	—	± VD	+~++	+
Glibenclamide	NS	± (VD ^b)	—	+~++ VD	+	±
Phloretin	NS	+	—	± VD	—	+
CFTR _{inh} -172	+++ VD	—/+~++ ^a	NS	+++	—	NS
CaCC _{inh} -A01	NS	—	NS	NS	++	NS
T16A _{inh} -A01	NS	—/+~++ ^a	NS	NS	++~+++	NS
GaTx1	—	NS	NS	+++	—	NS
GaTx2	+++	NS	NS	—	—	NS
Methadone	+++	NS	NS	—	NS	NS

Insensitivity and sensitivity are denoted by minus and plus signs, respectively, as follows: —, insensitive; ±, sensitive at sub-millimolar ($100 \leq \text{IC}_{50} < 1000 \mu\text{M}$) concentrations; +, sensitive at deca-micromolar ($10 \leq \text{IC}_{50} < 100 \mu\text{M}$) concentrations; ++, sensitive at micromolar ($1 \leq \text{IC}_{50} < 10 \mu\text{M}$) concentrations; and +++, sensitive at sub-micromolar ($\text{IC}_{50} < 1 \mu\text{M}$) concentrations. NS, not studied; VD, voltage-dependent block.

^aDepending on cell type (see the text for details).

^bVoltage-dependent block only by the charged form.

(Cuppoletti et al., 2013). The blockage mechanism is unclear but is different from the Cd^{2+} action and is not mediated by opioid receptors (Cuppoletti et al., 2013). Methadone was ineffective for CFTR (Cuppoletti et al., 2013), whereas its effects on other types of anion channels have not been studied. A thiazolidinone, CFTR_{inh}-172, was found to block not only CFTR (Ma et al., 2002; Kopeikin et al., 2010; Cuppoletti et al., 2014) but also ClC-2 with an IC_{50} of 143 nM in a voltage-dependent manner (Cuppoletti et al., 2014). In contrast, ClC-2 was not sensitive to stilbene-derivative anion channel blockers, 4-acetamido-4'-isothiocyano-2,2'-disulfonic stilbene (SITS) and 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) (Thiemann et al., 1992; Furukawa et al., 1998; Schwiebert et al., 1998; Xiong et al., 1999; Duan et al., 2000; Thompson et al., 2005), a DPC derivative niflumic acid (NFA) (Clark et al., 1998), tamoxifen (Duan et al., 2000), 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (DCPIB) (Decher et al., 2001), and Gd^{3+} (Chesnoy-Marchais and Fritsch, 1994; Tokimasa and North, 1996).

B. Volume-Sensitive Outwardly Rectifying Anion Channel Blockers

The most effective and selective VSOR blocker is DCPIB, which is an ethacrynic acid derivative, indanone compound (Decher et al., 2001). Its IC_{50} values were reported to be 3.5–4.1 μM in calf bovine pulmonary artery endothelial cells (Decher et al., 2001), 2.3 μM in mouse astrocytes (Liu et al., 2009), and approximately 3.5 μM in human bronchial epithelial cells (Stott et al., 2014) (see Table 2). DCPIB blocks VSOR in a voltage-independent and fully reversible manner (Decher et al., 2001). This compound was shown to be ineffective in blocking any other anion channels, including ClC-1/2/4/5/K1, CaCC, and CFTR (Decher et al., 2001) as well as Maxi-Cl (Sabirov and Okada, 2005; Sabirov et al., 2016) and ASOR (Sato-Numata et al., 2016). However, DCPIB can also block some K^+ channels (Deng et al., 2016), connexin 43 hemichannels (Ye et al., 2009), and gastric proton pump H^+/K^+ -ATPase (Fujii et al., 2015). Interestingly, a KCC antagonist, *R*-(+)-[(2-*n*-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)-oxy] acetic acid (DIOA) inhibits VSOR in rat thymocytes (Kurbannazarova et al., 2011) and blocks VSOR in HeLa cells with an IC_{50} of 20.9 μM (Sato-Numata et al., 2016). DIOA is not as specific as DCPIB and also inhibits ASOR with approximately 10 times higher efficiency (Sato-Numata et al., 2016). Structurally, both compounds are similar to a nonspecific Cl^- channel blocker, indanyloxyacetic acid 94, and contain a 6,7-dichloro-2-cyclopentyl-1-oxo-indanyl core with an oxy-carbonic acid (oxyacetate in DIOA and indanyloxyacetic acid 94, and oxybutyrate in DCPIB) connected to oxo-indanyl at position 5 (Fig. 9A). One may suggest that a short-chain oxy-carbonic acid combined with a chlorinated cyclopentyl-oxo-indanyl fragment represents a structural determinant for VSOR

block. We note that both DCPIB and DIOA also contain an *n*-butyl radical connected to the oxo-indanyl at position 2 (the same place as cyclopentyl); therefore, the only difference between DCPIB and DIOA is the length of the oxy-carbonic acid, which is slightly longer (by $-\text{CH}_2-\text{CH}_2-$) in DCPIB (Fig. 9A). This slight difference may provide greater specificity for DCPIB over DIOA.

A bisphenol, phloretin, also blocks VSOR, in a voltage-independent and fully reversible manner, only from the extracellular side with an IC_{50} of approximately 30 μM , without affecting time-dependent inactivation kinetics at large positive potentials (Fan et al., 2001). Similar to DIDS and SITS, phloretin contains two aromatic benzene rings but lacks the negative charge (Fig. 9B), which may explain the voltage-independent blocking. Phloridzin, which is a 2'- β -D-glucopyranoside-derivative of phloretin, was absolutely ineffective, suggesting that the hydroxyl group at the ortho-position of the carbonyl fragment as well as the overall shape and presumably hydrophobicity are important structural determinants for exerting channel inhibition (Fan et al., 2001). CaCC was insensitive to phloretin, whereas CFTR was sensitive to this compound at higher concentrations (IC_{50} = 195 and 355 μM at -60 and $+60$ mV, respectively) (Fan et al., 2001). Maxi-Cl is insensitive to phloretin (Sabirov et al., 2001; Dutta et al., 2002; Liu et al., 2006, 2008b). However, ASOR was voltage-independently sensitive to phloretin with an IC_{50} of 17.5 μM (Wang et al., 2007). An acidic di-aryl-urea, *N*-[3,5-bis(trifluoromethyl)-phenyl]-*N*-[4-bromo-2-(1*H*-tetrazol-5-yl)-phenyl] urea (NS3728), was reported to block VSOR with high affinity (IC_{50} = 0.30–0.46 μM ; Hélix et al., 2003; Klausen et al., 2007) in a manner independent of voltage (Hélix et al., 2003). However, CaCC was also very sensitive to NS3728 (Klausen et al., 2007) with an IC_{50} of 1.3 μM (Sauter et al., 2015). Since the sensitivity of ClC-2, Maxi-Cl, CFTR, and ASOR to NS3728 has not yet been examined, the specificity of this compound remains questionable.

VSOR is known to be sensitive to a number of conventional Cl^- channel blockers (see Okada, 1997). The stilbene-derivative blockers, SITS and DIDS, can inhibit VSOR in a voltage-dependent manner (Kubo and Okada, 1992; Nilius et al., 1994a; Shen et al., 1996). The reported IC_{50} values for SITS at positive and negative voltages are 1.5 and 6 μM , respectively, in Intestine 407 cells (Kubo and Okada, 1992), whereas those for DIDS are 22.7 and 88.5 μM in HeLa cells (Fig. 10), 0.84 and 11 μM in canine colonic myocytes (Dick et al., 1999), 26 and 256 μM in HEK293 cells (Hélix et al., 2003), 19.8 and 97.4 μM in normal human bronchial epithelial cells, and 9.0 and 81.4 μM in human cystic fibrosis bronchial epithelial cells (Stott et al., 2014), respectively. Such voltage-dependent blocking effects may be caused by open-channel blocking, because the size of divalent anion SITS (effective radius approximately 0.55 nm; Okada et al., 2009b) and DIDS

Fig. 9. Chemical structures of some VSOR blockers. (A) Etacrynic acid derivatives. The blue dashed line depicts the 2,3-dichlorophenoxy fragment, which is identical for all of these molecules. The red dashed line delineates a fragment (short-chain oxy-carbonic acid combined with chlorinated cyclopentyl-oxo-indanyl) common for VSOR blockers (see the text for details) but not for etacrynic acid. (B) VSOR blockers that have two aromatic rings connected with a chain of one to four atoms (carbon, nitrogen, or mixed). The blue dashed lines mark the first aromatic ring (see the text for details). IAA-94, indanyloxyacetic acid 94.

(approximately 0.54 nm; Okada et al., 2009b) is close to the cut-off radius of the VSOR pore (approximately 0.63 nm; Ternovsky et al., 2004). Although DIDS can inhibit Maxi-Cl and ASOR as well (see below), this inhibition was not dependent on voltages. Open-channel blocking of VSOR is also induced by extracellular, but not intracellular, application of negatively charged ATP with an IC_{50} of 2–5 mM at positive potentials (Nilius et al., 1994b; Tsumura et al., 1996), suggesting that ATP enters the pore only from its extracellular end to reach the ATP binding site inside the channel pore. A sulfonylurea, glibenclamide, exerts blocking actions on VSOR at positive voltages from the extracellular (not intracellular) side at physiologic pH by mixed mechanisms produced by charged and uncharged forms ($IC_{50} = 232 \mu\text{M}$ at pH 7.5): its uncharged form voltage-independently inhibits VSOR with an IC_{50} of 2.8 μM at pH 5, whereas the negatively charged form voltage-dependently inhibits VSOR with an IC_{50} of 532 μM at pH 9.2 (Liu et al., 1998). It must be noted that the effective radii of ATP^{4-} (approximately 0.58 nm) and glibenclamide⁻ (approximately 0.6 nm) are close to the VSOR pore size (Okada et al., 2009b). A hexavalent anion, suramin, also exhibits a similar voltage-dependent blocking action for VSOR currents with IC_{50} values of 1.5 μM in nasopharyngeal CNE-2Z cells (Yang et al., 2015) and 8.3 μM in HeLa cells (Sato-Numata et al., 2016) at positive voltages. The effective

radius of a minor axis (approximately 0.7 nm) of oval-shaped suramin is again close to the VSOR pore size (Okada et al., 2009b).

Carboxylate analogs [NPPB, DPC, and 9-AC] as well as DPC derivatives [NFA and flufenamic acid (FFA)] are also classified into conventional Cl^- channel blockers and inhibit VSOR in a voltage-independent manner (Okada, 1997). The IC_{50} values reported for NPPB are 25 μM in Intestine 407 cells (Kubo and Okada, 1992), 35–37 μM in rat inner medullary collecting duct cells (Boese et al., 1996), 21 μM in HEK293 cells (Hélix et al., 2003), 30.2 μM in human bronchial epithelial cells (Stott et al., 2014), 36.6 μM in cystic fibrosis bronchial epithelial cells (Stott et al., 2014), and 68 μM in astrocytoma cells (Bakhramov et al., 1995), whereas those for DPC and NFA are 350 μM (Kubo and Okada, 1992) and 320 μM (Sato-Numata et al., 2016), respectively.

In comparing the structures of phloretin, DIDS, SITS, DPC, NFA, FFA, NPPB, NS3728, and glibenclamide, one may notice that all of them have two aromatic rings connected with a chain of 1–4 atoms (carbon, nitrogen, or mixed) (Fig. 9B). This motif may represent another important structural determinant of VSOR blockers.

VSOR activity was sensitive, in a manner independent of membrane potentials, to a large variety of other structurally unrelated drugs, including a

Fig. 10. Voltage-dependent inhibition by DIDS of volume-sensitive outwardly rectifying Cl^- currents in human cervical carcinoma HeLa cells. (A) Representative current traces before and after application of DIDS. Step pulses were applied from -100 to $+100$ mV in 20 -mV increments, with a prepulse of -100 mV and a postpulse of -60 mV. (B) Concentration dependence of DIDS on the currents recorded at $+100$ mV (filled circles) and -100 mV (open circles) ($n = 3-6$). The IC_{50} values at $+100$ and -100 mV were 22.7 and 88.5 μM , respectively. Experimental conditions are the same as reported previously (Shimizu et al., 2004).

polyunsaturated fatty acid, arachidonic acid ($\text{IC}_{50} = 8$ μM ; Kubo and Okada, 1992); oxonol dyes, bis-(1,3-dibutylbarbituric acid)pentamethine oxanol (diBA-(5)- C_4) ($\text{IC}_{50} = 1.8$ μM ; Arreola et al., 1995) and WW781 [4-[4-[(1*E*,3*E*)-5-(1,3-dibutyl-2,4,6-trioxo-1,3-diazinan-5-ylidene)penta-1,3-dienyl]-3-methyl-5-oxo-4*H*-pyrazol-1-yl]benzenesulfonic acid] ($\text{IC}_{50} = 25$ μM ; Behe et al., 2017); an antimalaria drug, mefloquine ($\text{IC}_{50} = 1.2$ μM ; Maertens et al., 2000); a hemichannel blocker, carbenoxolone (CBX) ($\text{IC}_{50} = 15.4$ μM ; Benfenati et al., 2009); and cell-permeable CFTR inhibitors, GlyH-101 ($\text{IC}_{50} = 5$ to 6 μM ; Melis et al., 2014) ($\text{IC}_{50} = 9.5$ μM ; Friard et al., 2017) and PPQ-102 [6,7-dihydro-7,9-dimethyl-6-(5-methyl-2-furanyl)-11-phenylpyrimido[4',5':3,4]pyrrolo[1,2-*a*]quinoxaline-8,10(5*H*,9)-dione] ($\text{IC}_{50} = 19.6$ μM ; Friard et al., 2017). Inhibition of VSOR was also reported to be induced by a purinergic P2X receptor blocker, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (Galletta et al., 1997; Darby et al., 2003; Poletto Chaves and Varanda, 2008), in a voltage-dependent manner and by unprotonated quinidine in a voltage-independent manner (at pH 9; Voets et al., 1996; Behe et al., 2017). Since the drugs mentioned in this paragraph have no obvious common structural motives, we assume that VSOR channel protein has several pockets (binding sites) of different shapes and electrostatics to accommodate chemical substances of very different structures. It is also possible that some

of these drugs affect the regulatory auxiliary proteins but not the channel itself.

The VSOR currents were found to be voltage-dependently inhibited by a tyrosine kinase inhibitor, genistein (Bryan-Sisneros et al., 2000; Shuba et al., 2000; Inoue et al., 2007), but not by its inactive analog daidzein (Inoue et al., 2007). An antiestrogen, tamoxifen, which is a known inhibitor of P-glycoprotein, was found to voltage-independently inhibit VSOR activity in a number of nonexcitable cells (Zhang et al., 1994; Tominaga et al., 1995b; Meyer and Korbacher, 1996; Wondergem et al., 2001; Chen et al., 2002; Hélix et al., 2003; Yang et al., 2015) and colonic smooth muscle cells (Dick et al., 1999). In contrast, tamoxifen was ineffective in inhibiting VSOR in neuronal (Leaney et al., 1997; Inoue et al., 2005, 2007; Zhang et al., 2011) and muscular (Voets et al., 1997) cells as well as in some epithelial cells (Winpenny et al., 1996; Mitchell et al., 1997b) and neutrophils (Ahluwalia, 2008b). In contrast, it is noted that tamoxifen blocks some cationic channels in neuronal cells (Allen et al., 1998; Hardy et al., 1998) and muscular cells (He et al., 2003) as well as voltage-gated Ca^{2+} and K^+ channels in canine colonic myocytes at 10 μM (Dick et al., 1999). Since tamoxifen was found to inhibit NOX activity in human neutrophils (Ahluwalia, 2008b), there is a possibility that tamoxifen sensitivity of VSOR activities in some cell types is an indirect effect mediated by NOX inhibition (see Fig. 4;

type B mechanism). Other P-glycoprotein inhibitors, such as verapamil, nifedipine, 1,9-dideoxyforskolin, and quinidine, were reported to inhibit VSOR in some, but not all, cell types (see Okada, 1997),

VSOR activity was insensitive to heavy metal Cd^{2+} and Zn^{2+} (Okada et al., 2009b). Gd^{3+} was also ineffective at 30 μM for VSOR activity in many nonexcitable cells (Hazama et al., 1999, 2000; Sabirov et al., 2001; Liu et al., 2006) but sizably suppressed VSOR currents in canine colonic myocytes at 100 μM ($\text{IC}_{50} = 23 \mu\text{M}$; Dick et al., 1999). VSOR currents were largely insensitive to CaCC inhibitors, T16A_{inh}-A01 (2-[(5-ethyl-1,6-dihydro-4-methyl-6-oxo-2-pyrimidinyl)thio]-N-[4-(4-methoxyphenyl)-2-thiazolyl]-acetamide) and CaCC_{inh}-A01 [6-(1,1-dimethylethyl)-2-[(2-furanylcarbonyl)amino]-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid] in pancreatic ductal adenocarcinoma cells (Sauter et al., 2015), but they were recently reported to be sensitive to T16A_{inh}-A01 with an IC_{50} of 5.5 μM in HEK293 cells (Friard et al., 2017). CFTR_{inh}-172 was found to be ineffective in inhibiting VSOR in FRT cells (Ma et al., 2002) and in HEK293 cells (Friard et al., 2017) but was reported to voltage-independently inhibit VSOR in mouse kidney and hamster PS120 cells with IC_{50} values of 12 and 5.3 μM , respectively (Melis et al., 2014).

C. Maxi-Anion Channel Blockers

Heavy metal ion Gd^{3+} is the most effective blocker for Maxi-Cl (Sabirov et al., 2001, 2006; Bell et al., 2003; Dutta et al., 2004, 2008; Liu et al., 2006, 2008a; Toychiev et al., 2009; Kurbannazarova et al., 2011; Islam et al., 2012; Sabirov et al., 2017) with IC_{50} values of 0.75 μM in C127 cells (unpublished data) and 46 μM in L929 cells (Islam et al., 2012) (see Table 2). In addition, the blocking action was observed from outside (Fig. 11A), but not inside, the membrane (Sabirov et al., 2001; Toychiev et al., 2009) in a manner independent of voltage (Sabirov et al., 2001; Dutta et al., 2004). Gd^{3+} is ineffective in blocking other anion channels, including ClC-2 (Chesnoy-Marchais and Fritsch, 1994; Tokimasa and North, 1996), VSOR (Hazama et al., 1999, 2000; Sabirov et al., 2001; Liu et al., 2006; Okada et al., 2009b), CFTR (Hazama et al., 2000), CaCC (Waniishi et al., 1998), and ASOR (Sato-Numata et al., 2016), in many cell types. In contrast, however, Gd^{3+} was found to block a variety of nonanionic channels, including voltage-dependent Ca^{2+} channels (Docherty, 1988; Biagi and Enyeart, 1990; Dick et al., 1999), delayed K^+ channels (Dick et al., 1999), stretch-activated cation channels (Gustin et al., 1986; Yang and Sachs, 1989), Piezo1 channels (Coste et al., 2010), a number of transient receptor potential channel members (Zitt et al., 1996; Strotmann et al., 2000; Grimm et al., 2003; Hermosura et al., 2002; Hanano et al., 2004), and acid-sensing ion channels (Babinski et al., 2000). Another metal ion, Zn^{2+} , is also an effective blocker of Maxi-Cl (Schlichter et al., 1990; Kokubun et al., 1991;

Pahapill and Schlichter, 1992; O'Donnell et al., 2001). The detailed mechanisms for blocking of Maxi-Cl by Gd^{3+} and Zn^{2+} remain unclear.

Another effective blocker of Maxi-Cl is arachidonic acid (Riquelme and Parra, 1999; Dutta et al., 2002, 2004, 2008; Liu et al., 2006, 2008a; Sabirov et al., 2006, 2016; Islam et al., 2012) (Fig. 11A) with an IC_{50} of 4 to 5 μM (Dutta et al., 2002). However, arachidonic acid is also known to inhibit other anion channels such as VSOR (Kubo and Okada, 1992; Sato-Numata et al., 2016), CFTR (Linsdell, 2000; Zhou and Linsdell, 2007), and ASOR (Sato-Numata et al., 2016) with IC_{50} values of 3.5–50.5 μM . Maxi-Cl is inhibited by arachidonic acid itself (not by the downstream metabolic products) by two different mechanisms: one is channel shutdown with an IC_{50} of 4 to 5 μM , and the other is a unitary conductance reduction with an IC_{50} of 13 to 14 μM without affecting voltage-dependent changes in the open probability of Maxi-Cl. A negative charge and specific *cis*-conformation of the fatty acid chain are structural determinants important for blockage, which occurred only from the cytosolic side (Dutta et al., 2002). We recently identified SLCO2A1, known thus far as PGT, as the core component of Maxi-Cl and we found that it was very sensitive to PGT blocker BSP ($\text{IC}_{50} = 6.8\text{--}7.8 \mu\text{M}$) as well as to other PGT blockers bromocresol green and indocyanine green (Sabirov et al., 2017). BSP was effective from both sides of the membrane, producing a reversible, dose-dependent flickery blockage in a voltage-independent manner, which rules out the pore plug-in open-channel block mechanism.

Conventional anion channel blockers NPPB, SITS, and DIDS are also effective for Maxi-Cl, but their blocking effects are only partial (see reviews by Sabirov and Okada, 2004a, 2005, 2009; Sabirov et al., 2016). The Maxi-Cl current was voltage-independently blocked by NPPB with an IC_{50} of approximately 19 μM (Sun et al., 1992), as well as by SITS (Kokubun et al., 1991; Mitchell et al., 1997a; O'Donnell et al., 2001; Bell et al., 2003; Do et al., 2004; Liu et al., 2006; Islam et al., 2012) and by DIDS (Light et al., 1990; Sun et al., 1992; Brown et al., 1993; Riquelme and Parra, 1999) with an IC_{50} of approximately 100 μM only from outside the cell membrane (Groschner and Kukovetz, 1992).

Maxi-Cl activity was not virtually affected by a ClC-2 blocker, Cd^{2+} (1 mM), as shown in Fig. 12A. Maxi-Cl currents were observed to be insensitive to a variety of other anion channel blockers, including DCPIB (Sabirov et al., 2016) (Fig. 11B), phloretin (Sabirov et al., 2001; Dutta et al., 2004; Liu et al., 2006), 9-AC (Kokubun et al., 1991; O'Donnell et al., 2001), NFA (Sabirov et al., 2001), and CBX (De Marchi et al., 2008; Sabirov et al., 2016) (Fig. 11D). Maxi-Cl is also insensitive to pannexin hemichannel antagonists, probenecid, mefloquine, and $^{10}\text{Paxx1}$, as well as to connexin hemichannel antagonists, 1-octanol and gap junction peptide 27, GAP27, in

Fig. 11. Pharmacological profile of Maxi-Cl currents in patches excised from C127 cells. All of the columns represent the mean currents recorded at +25 mV. (A) Inhibitory effects of extracellular application of Gd^{3+} (50 μM) and intracellular application of arachidonic acid (20 μM). (B) Insensitivity to a VSOR blocker, DCPIB (10 μM), added from the intracellular or extracellular side. (C) Insensitivity to pannexin hemichannel antagonists, probenecid (1 mM) and 10 Panx1 (8 $\mu g/ml$), added to the extracellular (pipette) solution. (D) Insensitivity to gap junction antagonists, CBX (100 μM), 1-octanol (2 mM), and GAP27 (4 $\mu g/ml$), added to the extracellular (pipette) solution. Experimental conditions, other than the cell line used, are the same as reported previously (Islam et al., 2012; Sabirov et al., 2017). AA, arachidonic acid; Probe, probenecid.

L929 cells (Islam et al., 2012) and in C127 cells (Fig. 11, C and D). DPC was found to be ineffective for Maxi-Cl currents in B lymphocytes (Bosma, 1989), the placenta (Brown et al., 1993), and kidney macula densa cells (Bell et al., 2003) but was effective for Maxi-Cl currents in kidney cortical collecting duct cells (Light et al., 1990).

D. Cystic Fibrosis Transmembrane Conductance Regulator Blockers

The sulfonylureas glibenclamide and tolbutamide are effective blockers not only of ATP-sensitive potassium channels but also of CFTR. These negatively charged substances exert actions as open-channel blockers on CFTR by reducing open channel probability (Schultz et al., 1996, 1997; Venglarik et al., 1996; Sheppard and Robinson, 1997), with IC_{50} values of 2–38 μM for glibenclamide (Sheppard and Welsh, 1992; Rabe et al., 1995; Tominaga et al., 1995a; McNicholas et al., 1996; Schultz et al., 1996) and approximately 150 μM for tolbutamide (Sheppard and Welsh, 1992). However, glibenclamide was also known to block VSOR (Yamazaki

and Hume, 1997; Liu et al., 1998), CaCC (Yamazaki and Hume, 1997), and ASOR (Yamamoto and Ehara, 2006) at relatively higher concentrations. A diarylsulfonylurea, N(4-methylphenylsulfonyl)-N-(4-trifluoromethylphenyl)urea, DASU-02, is also an effective blocker for CFTR but not for ClC-2 (O'Donnell et al., 2000). A number of conventional anion channel blockers, including arylaminobenzoates (NPPB, DPC, and NFA), an aromatic carboxylic acid (9-AC), and disulfonic stilbenes (DIDS and DNDS), are also negatively charged and effective in blocking CFTR in a voltage-dependent manner. Among them, NPPB is most effective, exhibiting an IC_{50} value of 80 nM (Wangemann et al., 1986), although the blocking action of NPPB is nonspecific with blocking of all other VAACs and VCACs, except ASOR, at higher concentrations (see other parts of this section and Table 2). DPC and NFA can inhibit CFTR, although also nonspecifically and voltage-dependently. IC_{50} values for DPC are 237 μM at +100 mV and 912 μM at 0 mV (McCarty et al., 1993), whereas the IC_{50} for NFA

Fig. 12. Cd²⁺ insensitivity of Maxi-Cl (A) and ASOR (B) currents. (A) Representative Maxi-Cl currents recorded in inside-out patch membranes excised from C127 cells in the absence (control) and presence (+Cd²⁺) of 1 mM Cd²⁺ added to the extracellular (pipette) solution during application of step pulses from 0 to ±50 mV in 10-mV increments (protocol shown at the top). The bottom panel shows mean (± S.E.M.) NP_o values measured at +25 mV in the absence and presence of Cd²⁺. There was no significant difference (at $P > 0.05$) between the two values. Experimental conditions are the same as reported previously (Islam et al., 2012; Sabirov et al., 2017). (B) Whole-cell ASOR currents recorded in HeLa cells in the absence and presence of 1 mM Cd²⁺ added to the bath solution. The upper panel shows whole-cell currents before (pH 7.5) and during (pH 4.5) exposure to acidic bath solution in the absence and presence of Cd²⁺. The currents were elicited by application of alternating pulses from 0 to ±40 mV or of step pulses (at a, b, and c) from -100 to +100 mV in 20-mV increments. The middle panel shows expanded traces of current responses (at a, b, and c) to the step pulses. The bottom panels show $I-V$ relationships for the whole-cell current densities at pH 7.5 (squares) and pH 4.5 in the absence (circles) and presence (triangles) of Cd²⁺. There was no significant difference (at $P > 0.05$) between the circles and triangles at given voltages. Experimental conditions are the same as reported previously (Sato-Numata et al., 2016). NP_o , number of open channels.

is 253 μ M at -50 mV (Scott-Ward et al., 2004). 9-AC was reported to block CFTR from the extracellular side of cell membranes with IC₅₀ values of 942 μ M at -100 mV in guinea pig ventricular cells (Zhou et al., 1997) and 2.55, 1.70, and 1.26 mM at -60, -80 and -100 mV, respectively, in CFTR-transfected 3T3 cells (Ai et al., 2004), whereas 9-AC rather exhibited an enhancing effect on cardiac CFTR activity from the intracellular side by inhibiting some protein phosphatases (Zhou et al., 1997). DIDS and DNDS inhibit CFTR only from the cytosolic side in a manner dependent on voltages with an IC₅₀ of 236 and 80 μ M, respectively, at 0 mV (Linsdell and Hanrahan, 1996; also see Schultz et al., 1999). It is deemed that these negatively charged blockers enter the pore from its cytoplasmic end to reach the positively charged binding site, especially at lysine residue K95 in TM1, inside the channel pore (Linsdell, 2005, 2014; Zhou et al., 2010).

So-called more potent CFTR blockers have recently become available. A thiazolidinone, CFTR_{inh}-172,

voltage-independently inhibits CFTR activity in FRT cells overexpressing CFTR with an IC₅₀ of 300 nM (Ma et al., 2002) and in HEK293 cells overexpressing CFTR with an IC₅₀ of 133.6 nM (Cuppoletti et al., 2014). In addition, CFTR_{inh}-172 was found to inhibit human, but not shark, CFTR activity in the oocyte expression system with an IC₅₀ of 10.4 μ M (Stahl et al., 2012). Arginine 347 (R347), which does not contribute to CFTR pore formation, was identified to be a key position for the blocking action of CFTR_{inh}-172 (Caci et al., 2008; Zegarra-Moran and Galletta, 2017). However, it is noted that CFTR_{inh}-172 was found to inhibit ClC-2 equipotently (IC₅₀ = 143 nM) (Cuppoletti et al., 2014) and also VSOR in some cell types (Melis et al., 2014) but not in other cell types (Ma et al., 2002; Friard et al., 2017). A glycine hydrazide, GlyH-101, voltage-dependently suppresses epithelial CFTR activity with IC₅₀ values of 1.4 and 5.6 μ M at +60 and -60 mV, respectively, from the extracellular side

(Muanprasat et al., 2004) and cardiac CFTR activity with IC_{50} values of 0.3 and 5.1 μM at +100 and -100 mV, respectively (Barman et al., 2011). CFTR was very sensitive in a manner independent of voltage to a pyrimido-pyrrolo-quinoxalinedione, PPQ-102, with an IC_{50} of approximately 90 nM (Tradtrantip et al., 2009) and to a benzopyrimide-pyrrolo-oxazinedione, BPO-27, with IC_{50} values of approximately 8 nM (Snyder et al., 2011) and 0.5 and 360 nM from the intracellular and extracellular sides, respectively, by competition with ATP (Kim et al., 2015). A scorpion *Leiurus quinquestriatus hebraeus* venom peptide toxin, GaTx1, also voltage-independently blocks CFTR very potently from the intracellular side with an IC_{50} of 48 and 220 nM in the presence of intracellular ATP at 0.2 and 1 mM, respectively, without affecting ClC-2 and CaCC (Fuller et al., 2007).

CFTR currents are suppressed by other various substances, including the following: arachidonic acid, in a voltage-independent manner, with an IC_{50} of 3.5–6.5 μM from the intracellular side (Linsdell, 2000; Zhou and Linsdell, 2007); suramin, in a voltage-dependent manner, with an IC_{50} of 2.8 μM at +40 mV and 1 μM at -70 mV from the intracellular side (Bachmann et al., 1999); and phloretin, with IC_{50} values of 195–252 μM at -60 mV and 355–475 μM at +60 mV from the extracellular side (Fan et al., 2001). Components of the antidiarrheal Chinese herb *Regel Maxime*, (-)-epigallocatechin-3-gallate and (-)-epicatechin-3-gallate, were also reported to inhibit CFTR activity (Chen et al., 2015).

CFTR current was insensitive to a number of known anion channel blockers, including Cd^{2+} (Wang et al., 2009; Infield et al., 2016), Gd^{3+} (Hazama et al., 2000), DCPIB (Decher et al., 2001), methadone (Cuppoletti et al., 2013, 2014), and mefloquine (Maertens et al., 2000). In contrast to GaTx1, GaTx2 failed to inhibit CFTR activity (Thompson et al., 2009).

CFTR activity has been shown to be enhanced, but not suppressed, by several compounds. A flavonoid, genistein, which is a known potent PTK inhibitor, can PTK-independently activate CFTR from the extracellular (not intracellular) side (French et al., 1997; Wang et al., 1998; Zhou et al., 1998; Niisato et al., 1999). CFTR was also activated by some herbs such as menthol (Morise et al., 2010), which is a cyclic terpene alcohol produced by the peppermint herb; mashiningan (Harada et al., 2017), which is a Japanese herbal (Kampo) medicine used to treat constipation; as well as Junchoto, another Kampo medicine empirically prescribed for chronic constipation (Numata et al., 2018).

E. Calcium-Activated Chloride Channel Blockers

CaCC activity was reported to be blocked by a large variety of chemical substances (Frings et al., 2000). Among them, NFA is the most effective blocker for CaCC (see Table 2). Micromolar IC_{50} values were observed in vascular smooth muscle cells (6.6 μM in

Hogg et al., 1994b; 2 μM in Greenwood and Large, 1995), epithelial cells (7.6 μM in Qu et al., 2003a; approximately 2.5 μM in Romanenko et al., 2010), and melatonin-secreting pinealocytes (2.6 μM in Yamamura et al., 2018) as well as in TMEM16A-overexpressing Chinese hamster ovary (CHO) cells (7.4 μM in Liu et al., 2015). Deca-micromolar (10–99 μM) IC_{50} values were observed in *Xenopus* oocytes (17 μM in White and Aylwin, 1990; 10.1 μM in Qu and Hartzell, 2001), dog and cow tracheal epithelial cells (20 μM in Chao and Mochizuki, 1992), frog olfactory neurons (44 μM in Kleene, 1993), guinea pig tracheal smooth muscle cells (10 μM in Henmi et al., 1996), and rat vascular smooth muscle cells (26 μM in Robertson, 1998) as well as in TMEM16A-overexpressing HEK293 cells (12 μM in Bradley et al., 2014), whereas a sub-millimolar IC_{50} value was found in coronary smooth muscle cells (159 μM in Ledoux et al., 2005). However, NFA can also block VSOR with an IC_{50} of 320 μM in HeLa cells (Sato-Numata et al., 2016) and 55 μM in HEK293 cells (Friard et al., 2017) as well as ASOR with an IC_{50} of 11 μM in HeLa cells (Sato-Numata et al., 2016).

Although FFA, which was initially identified as an anti-inflammatory agent (Winder et al., 1963), is commonly used to block nonselective cation channels, it is also an effective CaCC blocker, exhibiting decamimolar IC_{50} values (20–60 μM) in native non-neuronal cells (White and Aylwin, 1990; Chao and Mochizuki, 1992; Greenwood and Large, 1995) and in TMEM16A-overexpressing CHO cells (Liu et al., 2015) but with an IC_{50} of 108 μM in frog olfactory neurons (Kleene, 1993).

CaCC activity is also sensitive to a number of, although not all, conventional anion channel blockers. DIDS voltage-dependently suppresses CaCC currents with deca-micromolar IC_{50} values (16.5–48 μM) in some native cells (Baron et al., 1991; Qu and Hartzell, 2001; Romanenko et al., 2010) or with sub-millimolar IC_{50} values (100–750 μM) in other native cells (Akasu et al., 1990; Wladkowski et al., 1998; Hogg et al., 1994a) as well as in *Tmem16a*-transfected CHO cells (Liu et al., 2015). Another stilbene-derivative (SITS) is a less potent blocker for CaCC, exhibiting an IC_{50} of 300–2000 μM (Akasu et al., 1990; Hussy, 1991; Wang et al., 1992; Hogg et al., 1994a). NPPB inhibits CaCC currents with an IC_{50} of 22–68 μM in *Xenopus* oocytes (Wu and Hamill, 1992) and 64.1 μM in CHO cells stably expressing TMEM16A (Liu et al., 2015). DPC was found to block CaCC with sub-millimolar IC_{50} values (111–320 μM) in several cell types (Baron et al., 1991; Chao and Mochizuki, 1992; Qu and Hartzell, 2001). 9-AC also inhibits CaCC with deca-micromolar to sub-millimolar IC_{50} values of 18.3 μM (Qu and Hartzell, 2001), approximately 30 μM (Romanenko et al., 2010), 117 μM (Baron et al., 1991), and 650 μM (Hogg et al., 1994a) and with an IC_{50} of 58 μM in HEK293 cells overexpressing TMEM16A (Bradley et al., 2014).

More potent CaCC blockers were recently identified by high-throughput screening (De La Fuente et al., 2008; Namkung et al., 2011). T16A_{inh}-A01 voltage-independently inhibits CaCC with a low micromolar IC₅₀ value (3.4 μ M) in rabbit urethral interstitial cells of Cajal (Fedigan et al., 2017) and with sub-micromolar to micromolar IC₅₀ values in TMEM16A-overexpressing cells (1.1 μ M in Namkung et al., 2011; 1.5 μ M in Bradley et al., 2014; 0.31 μ M in Liu et al., 2015). CaCC_{inh}-A01 also voltage-independently blocks CaCC with micromolar IC₅₀ values in both native cells (1.2 μ M in Fedigan et al., 2017) and TMEM16A-overexpressing cells (2.1 μ M in Namkung et al., 2011; 1.7 μ M in Bradley et al., 2014; 7.8 μ M in Liu et al., 2015). Although both CaCC antagonists were reported to fail to suppress VSOR activity in pancreatic ductal adenocarcinoma (Sauter et al., 2015), the specificity of these inhibitors has not been well studied, especially in other cell types, and thus remains questionable (see Table 2). In fact, T16A_{inh}-A01 was recently found to block not only L-type voltage-gated Ca²⁺ channels with an IC₅₀ of 0.05 μ M in vascular smooth muscle A7r5 cells (Boedtker et al., 2015) but also VSOR with an IC₅₀ of 5.5 μ M in HEK293 cells (Friard et al., 2017). In addition, CaCC_{inh}-A01 (10 μ M) increased the intracellular Ca²⁺ level in HEK293 cells (Kunzelmann et al., 2012).

Other potent CaCC blockers include tannic acid, *N*-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid, and 2-(4-chloro-2-methylphenoxy)-*N*-[(2-methoxyphenyl)-methylideneamino]-acetamide. The IC₅₀ values of tannic acid for CaCC inhibition were reported to be 3.1 μ M in T84 cells and 11.2 μ M in human bronchial epithelial cells (Namkung et al., 2010) and 25.68 μ M in stably *Tmem16a*-transfected CHO cells (Liu et al., 2015). *N*-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid was found to more potently block CaCC currents in *Xenopus* oocytes, in which xTMEM16A is endogenously expressed with an IC₅₀ of 0.08 μ M and in transiently hTMEM16A-expressing HEK293 cells with an IC₅₀ of 1.27 μ M without affecting CFTR and ClC-2 currents at 10–30 μ M (Oh et al., 2013). 2-(4-Chloro-2-methylphenoxy)-*N*-[(2-methoxyphenyl)methylideneamino]-acetamide was observed to completely and voltage-independently inhibit CaCC currents in normal human nasal epithelial cells and hTMEM16A-overexpressing FRT cells with sub-micromolar potency (IC₅₀ = 0.11 μ M) without inhibiting mClC-2 and hCFTR currents at 10 μ M (Seo et al., 2016).

A putative VSOR blocker, NS3728, was found to equipotently block CaCC with IC₅₀ values of 2.1 μ M at –95 mV and 0.7 μ M at +50 mV in ELA cells (Klausen et al., 2007) and 1.3 μ M in TMEM16A-overexpressing HEK293 cells (Sauter et al., 2015). CaCC activity was also sensitive to known CFTR blockers, glibenclamide and GlyH-101. IC₅₀ values of glibenclamide for CaCC blocking were 61.5 μ M at +50 mV and 69.9 μ M at –100 mV in cardiac myocytes (Yamazaki and Hume, 1997), and the IC₅₀ of GlyH-101 was 3.4 μ M in kidney

tubular cells (Melis et al., 2014). In contrast, another CFTR blocker, CFTR_{inh}-172, was observed to be ineffective in blocking native CaCC currents at ≥ 5 μ M in kidney tubular cells (Melis et al., 2014) and TMEM16A-associated CaCC currents in stable-transfected FRT cells at 5–100 μ M (Caputo et al., 2008). CaCC activity was reported to be insensitive to VSOR blocker DCPIB (Decher et al., 2001), Maxi-Cl blocker Gd³⁺ (Waniishi et al., 1998), and phloretin (Fan et al., 2001), which is an effective blocker for VSOR (Fan et al., 2001) and ASOR (Wang et al., 2007; Sato-Numata et al., 2014). Although tamoxifen was found to be inhibitory for CaCC solely in the calf bovine pulmonary artery endothelial cell line (Nilius et al., 1997b), subsequent studies failed to show any inhibitory effect on CaCC activity in human pancreatic ductal adenocarcinoma HPAF cells (Winpenny et al., 1998) and in mouse kidney inner medullary collecting duct cells (Qu et al., 2003a).

F. Acid-Sensitive Outwardly Rectifying Anion Channel Blockers

The most effective blocker of ASOR currently available is DIDS applied from the extracellular site. The IC₅₀ values of DIDS for ASOR were reported to be sub-micromolar (0.12 μ M in Wang et al., 2007; 0.5 μ M in Capurro et al., 2015) or micromolar (2.9 μ M in Lambert and Oberwinkler, 2005; 4.7 μ M in Sato-Numata et al., 2014) (see Table 2). However, it is noted that DIDS can also block VSOR, Maxi-Cl, and CaCC, although at higher concentrations, as described above. Blocking effects of DIDS on ASOR and Maxi-Cl are voltage independent, whereas those on VSOR and CaCC are voltage dependent. ASOR activity was highly sensitive to suramin and DIOA with IC₅₀ values of 0.05 μ M and 1.9 μ M, respectively (Sato-Numata et al., 2016). Notably, suramin blocked ASOR at concentrations two orders of magnitude lower than VSOR and CFTR, and this blocking action was exerted in a voltage-independent manner, in contrast to its voltage-dependent blocking action on VSOR (Sato-Numata et al., 2016) and CFTR (Bachmann et al., 1999). In addition, ASOR activity was suppressed by NFA and arachidonic acid with IC₅₀ values of 11.0 μ M and 8.9 μ M, respectively (Sato-Numata et al., 2016). It is worth mentioning that ASOR is one to two orders of magnitude more sensitive to NFA and arachidonic acid as well. ASOR is also inhibited by phloretin with an IC₅₀ of 17.5–22.0 μ M (Wang et al., 2007; Sato-Numata et al., 2014) and glibenclamide with an IC₅₀ of approximately 100 μ M (Yamamoto and Ehara, 2006).

ASOR activity was not blocked by a ClC-2 blocker, Cd²⁺ (1 mM), as shown in Fig. 12B. ASOR currents were found to be insensitive to a number of other anion channel blockers, including DCPIB, NPPB, CBX, Gd³⁺, and mefloquine (Sato-Numata et al., 2016) as well as tamoxifen (Nobles et al., 2004; Yamamoto and Ehara, 2006; Sato-Numata et al., 2016). Extracellular application of ATP and a P2 receptor blocker,

pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (which are known to block VSOR), exerted no blocking action on ASOR (Sato-Numata et al., 2016).

G. Their Pharmacological Distinctions

Distinguishing VAACs and VCACs from each other must eventually, in principle, be done by investigating differences in their phenotypic biophysical properties and by observing the effects of abolition or modification of genes for their molecular identities. However, when specific and selective blockers are available, such pharmacological agents would provide the quickest and easiest tools to detect the involvement of particular channels in biologic events.

The selectivity and specificity of chemical substances for VAACs and VCACs are summarized by providing the IC_{50} values together with voltage dependence for their blocking action in Table 2. Based on the information listed in Table 2, the pharmacological distinctions between these anion channels can be summarized as follows. Cd^{2+} is the most selective blocker for ClC-2. However, effects of Cd^{2+} on CaCC activity have not been elucidated, maybe at least in part because Cd^{2+} often blocks channel-mediated Ca^{2+} entry, thereby affecting the intracellular Ca^{2+} level. To distinguish ClC-2 from CaCC, its GaTx2 sensitivity would provide additional evidence. Simply, DCPIB sensitivity can discriminate VSOR from the others. Micromolar sensitivity to Gd^{3+} may distinguish Maxi-Cl from the others. When the anion channel in question exhibits deca-micromolar to sub-millimolar sensitivity to Gd^{3+} , its insensitivity to DCPIB or phloretin provides a good indication for Maxi-Cl to discriminate from VSOR. CFTR_{inh}-172 is not specific to CFTR. Among the chemicals listed in Table 2, GaTx1 is likely the most potent blocker for CFTR. However, GaTx1 sensitivity of VSOR, Maxi-Cl, and ASOR has not yet been reported. To distinguish CFTR from the others, its sub-micromolar sensitivity to NPPB or micromolar sensitivity to glibenclamide is indispensably to be examined in combination with GaTx1 sensitivity. Although CaCC_{inh}-A01 and T16A_{inh}-A01 are purportedly expected to be CaCC-selective blockers, information about the sensitivity of ClC-2, Maxi-Cl, CFTR, and ASOR to both drugs is currently lacking. Furthermore, the sensitivity of VSOR to T16A_{inh}-A01 was reported in some, but not all, cell types. To distinguish CaCC from ClC-2, Maxi-Cl, CFTR, and ASOR, its micromolar sensitivity (if any) to NFA may thus provide additional evidence in combination with sensitivity to CaCC_{inh}-A01 and T16A_{inh}-A01. Sub-micromolar sensitivity to DIDS alone would provide supporting evidence for ASOR. Even when DIDS sensitivity ranges from micromolar to sub-millimolar concentrations, NPPB insensitivity may provide additional evidence for ASOR.

V. Conclusions and Future Directions

In relation to cell volume, there are two groups of anion channels: VAACs and VCACs. VAACs are directly activated by cell swelling and consist of inward-rectifier ClC-2, mild outward-rectifier VSOR (also called VRAC), and nonrectifying large-conductance Maxi-Cl. The molecular identities of core components of VSOR and Maxi-Cl were recently discovered by unbiased genome-wide approaches. However, some other as-yet-unknown regulatory components remain to be identified for whole VSOR and Maxi-Cl complexes. VCACs consist of cAMP-activated ohmic small-conductance CFTR activated by cAMP/PKA, strong outward-rectifier CaCC activated by cytosolic Ca^{2+} , and another strong outward-rectifier activated by acid. Although CFTR and CaCC molecules were successfully identified, the molecular basis of ASOR has not yet been elucidated. A large number of chemical compounds have been known to block VAACs and VCACs with respective sub-micromolar to sub-millimolar IC_{50} values. DCPIB is the sole selective blocker specific to VSOR. However, others exhibit, more or less, broad specificity or their specificity remains uncertain. Nevertheless, pharmacological distinctions of VAACs and VCACs from each other can be suitably attained by the combinatory application of two different blockers relatively specific to given anion channels. Discovery of more specific blockers requires future studies performed particularly on the basis of molecular understanding. Since these anion channels are prerequisitely involved not only in important physiologic functions but also in a variety of pathologic situations and human diseases such as ischemia/reperfusion injury and cancer, future findings of their highly specific blockers or activators would be beneficial for therapeutic purposes.

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Authorship Contributions

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