Letter to the Editor

Criteria for the Molecular Identification of the Volume-Sensitive Outwardly Rectifying Cl^- Channel

Yasunobu Okada, Shigetoshi Oiki, Akihiro Hazama, and Shigeru Morishima

From the Department of Cellular and Molecular Physiology, National Institute for Physiological Sciences, Okazaki 444-8585, Japan

Anion channels are activated by volume expansion in most animal cell types and are known to be implicated not only in regulatory volume decrease but also in many other cell activities that are associated with changes in cell volume and shape, including cell proliferation and cell death (Okada, 1998). Among a variety of swelling-activated Cl^- channels ($I_{Cl,swell}$), the most important and frequently observed channel is a volume-sensitive, outwardly rectifying, ATP-dependent Cl^- channel (Okada, 1997; termed VSOR-ClC here). The functional properties of VSOR-ClC have so far been intensively and systematically studied in rat C6 glioma cells (Strange et al., 1996), human epithelial Intestine 407 cells (Okada, 1997), and human and bovine endothelial cells (Nilius et al., 1997), and proved to share the same properties. The principal phenotypic properties are attributed to the pore and regulatory characteristics, as summarized in Table I. The regulatory properties of the channel may be modified by association with putative regulatory subunits. Thus, the "fingerprint" characteristics of the channel, which distinguish it from other Cl^- channels, should be related, at least, to the pore properties, such as its intermediate single-channel conductance, outward rectification, low-field anion selectivity, a high open probability (>0.95), voltage-dependent inactivation gating at large positive potentials, and open-channel block by extracellular ATP.

Although much work has been performed to identify the VSOR-ClC protein, the molecular identity of the channel has not as yet been determined. Purification of the channel protein has been hindered by the lack of a highly specific channel ligand (antagonist or agonist). Expression cloning of the VSOR-ClC protein has been hampered by the endogenous expression and housekeeping activity of the channel in almost all cell types (Nilius et al., 1994; Okada, 1997), including *Xenopus* oocytes (Ackerman et al., 1994), and by possible changes in volume sensitivity of the channel and/or the osmosensitivity of the cell produced by overexpression of exogenous proteins (Okada, 1997).

From gene expression studies, three candidates for the VSOR-ClC protein have been proposed: the MDR1 gene product, P-glycoprotein (PGP; Valverde et al., 1992; Gill et al., 1992), a ubiquitous (principally cytoplasmic) protein, pICln (Paulmichl et al., 1992), and a member of the cloned ClC family, ClC-3 (Duan et al., 1997). However, the PGP hypothesis is no longer viable, as reviewed by Okada (1997). The most crucial evidence against the PGP hypothesis was the observation that abolition of the endogenous PGP expression by antisense oligonucleotides failed to affect the VSOR-ClC current in Intestine 407 cells (Tominaga et al., 1995). The misleading conclusion in the original studies (Valverde et al., 1992; Gill et al., 1992) may have been caused by exaggerated experimental conditions, because overexpression of PGP was found to augment volume sensitivity of endogenous VSOR-ClC currents (Miwa et al., 1997). Now, the pICln hypothesis has also been discarded, as summarized recently in two letters for *Perspectives in General Physiology* (Strange, 1998; Clapham, 1998). Several lines of crucial evidence against the pICln hypothesis were provided by Voets et al. (1996, 1998), Buyse et al. (1997), and Emma et al. (1998). It should be stressed that overexpression of pICln in *Xenopus* oocytes resulted in activation of Cl^- currents with pore properties (rectification, anion selectivity, and blocking) distinct from the phenotypic properties of VSOR-ClC (Voets et al., 1996).

The ClC-3 hypothesis is at present viable, though not completely tested. As summarized in Table I, the pore properties, except the open probability, of guinea pig cardiac ClC-3-associated Cl^- currents (Duan et al., 1997) are largely similar to those of VSOR-ClC. Interestingly, the N579K mutant of guinea pig ClC-3 was found to bring about changes in anion selectivity from $I^- > Cl^-$ to $Cl^- > I^-$ and in rectification from outward to inward (Duan et al., 1997). Enormous basal activity under isotonic conditions and sensitivity to an activator of pro-

Address correspondence to Yasunobu Okada, Department of Cellular and Molecular Physiology, National Institute for Physiological Sciences, Okazaki 444-8585, Japan. Fax: 81-564-55-7735; E-mail: okada@nips.ac.jp

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TABLE I

Comparison of Properties of Human, Bovine, and Rat Volume-sensitive Outwardly Rectifying Cl^- Channels, Guinea Pig ClC-3-associated Cl^- Channels and Rat ClC-2-associated Cl^- Channels

	VSOR-ClC	ClC-3	ClC-2
Pore properties			
Single-channel conductance	Intermediate	Intermediate	Small
Open probability	>0.95	~0.6	?
Rectification	Outward	Outward	Inward
Gating property	Depolarization-induced inactivation	Depolarization-induced inactivation	Hyperpolarization-induced inactivation
Anion selectivity	I > Br > Cl	I > Cl	Cl ≥ Br > I
Open-channel block	Extracellular ATP	Extracellular ATP	?
Regulatory properties			
Basal activity	None	Enormous	Large
Volume sensitivity	Swelling-activated	Swelling-enhanced	Swelling-enhanced
PKC sensitivity	TPA, OAG-insensitive	PDBu-sensitive	?
Intracellular ATP dependence	ATP requirement	?	?

tein kinase C of the ClC-3-associated Cl^- currents are distinct from the properties of human, bovine, and rat VSOR-ClC currents. Therefore, there is a possibility that VSOR-ClC has a pore formed by ClC-3 and regulatory subunit(s) that may contain the volume sensor and PKC phosphorylation sites, and these regulatory subunits may differ between animal species. Alternatively, it is also possible that VSOR-ClC is formed by the ClC-3 isoform alone, the cytosolic regulatory domains of which may be different between animal species. For example, the NH_2-terminal 58 amino acid residues of the human and mouse ClC-3 isoforms are missing from the rat and guinea-pig isoforms. However, a definite conclusion concerning the contribution of ClC-3 to VSOR-ClC awaits further studies by knocking out endogenous ClC-3 expression and by heterologous expression of the human, bovine, and rat isoforms of ClC-3. In CHO cells stably expressing the rat kidney-derived ClC-3 isoform, quite distinct properties (such as maxi unitary conductance) of Cl^- currents were observed (Kawasaki et al., 1994). However, it should be noted that the inside-out patch-clamp study was performed in the absence of intracellular ATP, which is indispensable for normal activities of VSOR-ClC (Okada, 1997). Also, there remains the possibility that VSOR-ClC is formed by a heteroligomer of ClC-3 and other ClC family members or solely by the latter protein(s).

Another cloned Cl^- channel, ClC-2 (Thiemann et al., 1992), has also been listed as a candidate of VSOR-ClC because of its volume sensitivity and ubiquitous expression (Grunder et al., 1992). However, as summarized in Table I, the following pore properties of ClC-2 are quite distinct from VSOR-ClC: the small unitary conductance (3–5 pS; Jentsch et al., 1995), inward rectification, inactivation gating at negative potentials, and the anion selectivity sequence of $Cl^- \geq Br^- > I^-$ (Thiemann et al., 1992). Thus, although the possibility remains that ClC-2 may contribute to VSOR-ClC function, it is unlikely that VSOR-ClC is formed solely by ClC-2.

Taken together, it appears that we are still far from the identification of VSOR-ClC protein. To avoid unnecessary repetition of premature conclusions, as was the case with the PGP and pICln hypotheses, we propose the following criteria for the molecular identification of VSOR-ClC: (*a*) transfection with the gene for the candidate protein induces swelling-activated anionic currents with characteristics identical to those of phenotypic properties of VSOR-ClC, (*b*) the cells functionally exhibiting the VSOR-ClC current express endogenously the candidate mRNA and protein, (*c*) abolition of expression of the candidate protein abolishes the endogenous VSOR-ClC current, and (*d*) the mutation of the candidate gene gives rise to significant changes in the pore properties of the Cl^- current. Before obtaining evidence fulfilling all the above criteria, the molecular identity of VSOR-ClC cannot be established. This is what we have learned from previous work that evolved around the PGP and pICln hypotheses.

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