Distinct contributions of LRRC8A and its paralogs to the VSOR anion channel from those of the ASOR anion channel

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ABSTRACT
Volume- and acid-sensitive outwardly rectifying anion channels (VSOR and ASOR) activated by swelling and acidification exhibit voltage-dependent inactivation and activation time courses, respectively. Recently, LRRC8A and some paralogs were shown to be essentially involved in the activity and inactivation kinetics of VSOR currents in human colonic HCT116 cells. In human cervix HeLa cells, here, inactivation of VSOR currents was found to become accelerated by RNA silencing only of LRRC8A but never decelerated by that of any LRRC8 isoform. These data suggest that LRRC8A is associated with the deceleration mechanism of VSOR inactivation, while none of LRRC8 members is related to the acceleration mechanism. Activation kinetics of ASOR currents was unaffected by knockdown of any LRRC8 family member. Double, triple and quadruple gene-silencing studies indicated that combinatory expression of LRRC8A with LRRC8D and LRRC8C is essential for VSOR activity, whereas none of LRRC8 family members is involved in ASOR activity.

KEYWORDS
activation; anion channel; ASOR; inactivation; LRRC8; VRAC; VSOR

Introduction
The volume-sensitive outwardly rectifying anion channel (VSOR), often called volume-regulated anion channel (VRAC), is ubiquitously expressed and slowly activated by osmotic cell swelling1,2 or apoptotic stimulation,3 thereby being involved in cell volume regulation and apoptotic death induction.4,5 The VSOR current is phenotypically characterized by volume sensitivity, intermediate single-channel conductance, mild outward rectification, voltage-dependent inactivation kinetics, weak-field strength anion selectivity, non-hydrolytic dependence on intracellular ATP, sensitivity to intracellular free Mg\(^{2+}\), and open-channel block by extracellular ATP.6 Recently, the leucine-rich repeats containing 8A (LRRC8A) protein was identified as the essential component of VSOR by 2 independent research groups.7,8 Further studies from the same groups showed that functional VSOR currents require, in addition to LRRC8A, at least one of LRRC8 paralogs (LRRC8B/C/D/E).8,9 Moreover, LRRC8E and LRRC8C were found to play accelerating and decelerating roles, respectively, in voltage- and time-dependent inactivation of VSOR currents at large positive membrane potentials in human colonic tumor HCT116 cells.8,10 However, it is not known whether LRRC8A paralogs play similar roles in other cell types. The present study first addressed this question by applying RNA-silencing approaches to human cervical cancer HeLa cells in which the essential role of LRRC8A was also established.7,8,11

Another ubiquitously expressed anion channel, called acid-sensitive outwardly rectifying anion channel (ASOR), is rapidly activated by extracellular acidification,12,13 in a manner highly sensitive to temperature,14,15 and thereby involved in acidotoxic cell death.15,16 Although the molecular identity of VSOR has not been determined as yet, its phenotypic properties have been well characterized as acid

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sensitivity, strong outward rectification, weak-field anion permeability sequence, and activation kinetics upon application of positive voltages.13,16,17 Our recent study showed that siRNA-mediated knockdown of each LRRC8 family member alone does not essentially affect ASOR activity.11 However, it remains uncertain whether any LRRC8 member plays a regulatory role in its activation kinetics. Thus, this question was addressed in the second part of present study.

Results

**Effects of siRNA-mediated knockdown of each LRRC8 family member on inactivation kinetics of VSOR currents and activation kinetics of ASOR currents**

We previously found in HeLa cells that VSOR activity was immensely suppressed by siRNA-mediated knockdown of LRRC8A, but not that of any LRRC8A paralog.11 In contrast, ASOR activity was found to be not significantly affected by gene silencing for each LRRC8 isoform.11 In LRRC8A-siRNA-transfected HeLa (ΔA) cells, as shown in Fig. 1A, the amplitude of VSOR currents were markedly reduced, as found previously.7,8,11 In addition, depolarization-induced inactivation of VSOR currents became faster (upper panel) with significantly reducing the apparent half-inactivation time in ΔA cells (bottom panel), whereas the inactivation kinetics was never affected by transfection with siRNAs for other isoforms (ΔB–ΔE: bottom panel). On the other hand, as shown in Fig. 1B, activation kinetics of ASOR currents and the apparent half-activation time were not significantly affected by single gene-silencing for any LRRC8 isoform. These results indicate that the inactivation process of VSOR currents is, for some reason, facilitated by depletion of LRRC8A, but none of other LRRC8 isoforms contributes to the activation kinetics of ASOR currents.

In addition to the diminishing effects on the inactivation rate and the amplitude of VSOR currents,
depletion of LRRC8A affected the pattern of inactivation kinetics (Fig. 1A: upper right). The time course of VSOR inactivation is most appropriately fitted with a single-exponential function in ΔA cells, whereas that is with a double-exponential function in Control cells (see Legend for Fig. 1A and also Insets in Fig. 3). This fact suggests that the inactivation mechanism of VSOR is affected, in not only quantitative but also qualitative manners, by depletion of LRRC8A. In contrast, the activation kinetics of ASOR currents was not affected by depletion of LRRC8A (Fig. 1B: upper right) and is best fitted with a double-exponential function in both Control and ΔA cells (see Legend for Fig. 1B).

**Effects of multiple siRNA-mediated knockdown of LRRC8 family members on the activities of VSOR and ASOR**

In order to examine whether any combination of LRRC8 family members is essentially involved in the activity of VSOR or ASOR, we next observed the effects of gene silencing for multiple LRRC8 isoforms. Both outward and inward VSOR currents were markedly reduced not only in ΔA cells but also in HeLa cells transfected triply with siRNAs for LRRC8C, LRRC8D and LRRC8E (ΔCDE) or quadruply with those together with siRNA for LRRC8B (ΔBCDE), as shown in Fig. 2A. VSOR currents were less markedly, but significantly, suppressed in the cells doubly transfected with siRNAs of LRRC8D and LRRC8E (ΔDE) at ±100 mV or with those of LRRC8C and LRRC8E (ΔCE) at −100 mV (Fig. 2A). Since VSOR activity was strongly suppressed in ΔCDE cells but not in ΔBE cells, it is conceivable that combinatorial expression of LRRC8A together with LRRC8C and LRRC8D is most effective to maintain a robust VSOR activity in HeLa cells.

In contrast to VSOR currents, as shown in Fig. 2B, ASOR currents recorded at negative and positive voltages were not altered in ΔA, ΔCE, ΔDE, ΔCDE and
Discussion

Based on similarities in some pharmacological properties and the peak amplitudes between ASOR and VSOR currents, it was previously suggested that both types of outwardly rectifying anion channels share the same molecular entity. However, our recent study showed that ASOR currents in HeLa cells were not affected by a number of well-known VSOR blocking agents including DCPiB, NPPB, carbenoxolone, pyridoxalphosphated-6-azophenyl-2,4'-disulfonic acid (PPADS), meltoquine and tamoxifen, suggesting that the molecular basis for ASOR is different from that of VSOR. Actually, the ASOR current amplitude was not affected by siRNA-mediated knockdown of LRRC8A in HeLa cells, despite that LRRC8A was determined as an essential component of VSOR in this cell line. Furthermore, in the present study, activation kinetics of ASOR currents observed upon application of positive voltages was found to be unaffected by RNA silencing not only of LRRC8A but also of its paralog (Fig. 1B). In addition, ASOR activity was found to be unaffected by double knockdown of LRRC8C and 8D or that of LRRC8D and 8E, as well as by triple knockdown of LRRC8C, 8D and 8E, and by quadruple knockdown of LRRC8B, 8C, 8D and 8E (Fig. 2B). Thus, it is reasonable to conclude that the molecular entity of ASOR is independent of any LRRC8 family members.

The molecular identity of VSOR was highly controversial for decades, since its functional discovery in 1988 until recent identification of LRRC8A as the essential component. Studies performed in HCT116 cells showed that VSOR activity requires combinatorial expression of LRRC8A and at least one of the other 4 LRRC8 isoforms. The present gene-silencing study showed that robust VSOR activity in HeLa cells is most effectively maintained by combinatorial expression of LRRC8A with LRRC8C and LRRC8D (Fig. 2A). A recent reconstitution study demonstrated that the complex of LRRC8A with LRRC8D or LRRC8E is sufficient to form anion channels activated by hypotonicity or reduced ionic strength. However,
it must be pointed out that, in several fundamental properties, the reconstituted anion channel is distinct from VSOR. The reconstituted channel activity was not dependent on intracellular ATP, did not exhibit time-dependent inactivation upon application of positive voltages, and had a much smaller single-channel conductance. Thus, it seems likely that some as-yet-unidentified non-LRRC8 component(s), X, is additionally required to reproduce a whole set of the phenotypes of VSOR. Actually, combinatory overexpression of LRRC8A with any other isoform was found to fail to increase VSOR current amplitudes above wild-type values in HEK cells. Furthermore, our recent study indicated that deficiency of VSOR activity in cisplatin-resistant KCP-4 cells is due to aberrations of some component other than LRRC8 family members. Hence, it is likely that the complex of LRRC8A, LRRC8C, LRRC8D and X forms the core of VSOR pore in HeLa cells.

In the present study, depletion of LRRC8A, but not of any other isoform, was found to hasten the inactivation of VSOR currents in HeLa cells (Fig. 1A). This suggests that LRRC8A per se or its associated regulatory subcomponent serves as a decelerator for VSOR inactivation in HeLa cells. In contrast to the previous results obtained in HCT116 cells, we did not observe any effects of LRRC8C and LRRC8E knockdown on the VSOR inactivation kinetics in HeLa cells (Fig. 1A). Thus, it appears that molecular nature of accelerator and decelerator involved in the VSOR inactivation mechanism is different from each other depending on the cell types. This difference may be most simply explained by assuming that modulation of the VSOR inactivation involves some auxiliary components associated with the VSOR pore/gate proteins but not LRRC8 isoform(s) per se. Since siRNA-mediated knockdown of any single LRRC8 isoforms failed to retard the inactivation rate of VSOR in HeLa cells (Fig. 1A), it is reasonable to further assume that an as-yet-unidentified accelerating factor or subcomponent is associated with X of the VSOR pore complex in HeLa cells.

Based on the above considerations, Fig. 3 depicts a hypothetical model of the structure of the VSOR pore domain, which contains LRRC8A, in HeLa cells. Its hexameric structure is inferred on the basis of a molecular similarity of LRRC8 proteins to pannexins. More specifically, the hexameric VSOR pore is formed by the core complex of LRRC8A with LRRC8C, LRRC8D and X, and accessory accelerating and decelerating subcomponents (a and d: Fig. 3) are associated with X and LRRC8A, respectively. It is plausible that availability of the accessory accelerating subcomponent (a) is limited in control HeLa cells, and, therefore, fast-inactivating pores associated with a coexist with slow-inactivating pores without a (Fig. 3A). Under these conditions, the inactivation of whole-cell VSOR currents should exhibit a double-exponential time course (inset in Fig. 3A), since 2 distinct populations of open-state pores are present. When the total number of active pores was reduced by depletion of LRRC8A, such a limited number of a becomes sufficient to bind to all of the remaining LRRC8A-containing pores, giving rise to a uniform population of a-associated pores (Fig. 3B) that generate fast-inactivating currents with a monoeXponential time course (Inset in Fig. 3B).

Further studies should be required to verify this hypothetic model by which the precise molecular mechanism for VSOR inactivation will be unequivocally understood. On the other hand, the molecular mechanism of voltage- and time-dependent activation of ASOR should await its molecular identification in future.

Materials and methods
Cell culture of HeLa cells, modification of gene expression, and whole-cell patch-clamp experiments were performed as previously described. For double, triple or quadruple knockdown, cells were transfected simultaneously with multiple siRNAs for hLRRC8 family members. The apparent half-inactivation time of VSOR anion current was estimated by the time point, at which the inactivation reached half of the total inactivation after 1 s of step pulse at +100 mV. The apparent half-activation time of ASOR anion current was estimated by the time point, at which the activation reached half of the total activation after 600 ms of step pulse at +100 mV. Data are presented as means ± SEM of n observations. Statistical analyses were made by ANOVA-Bonferroni test.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.
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References
[1] Cahalan MD, Lewis RS. Role of potassium and chloride channels in volume regulation by T lymphocytes. Soc Gen Physiol 1988; 43:281-301
[2] Hazama A, Okada Y. Ca\(^{2+}\) sensitivity of volume-regulatory K\(^{-}\) and Cl\(^{-}\) channels in cultured human epithelial cells. J Physiol 1988; 402:687-702; PMID:2466988; http://dx.doi.org/10.1113/jphysiol.1988.sp017229
[3] Shimizu T, Numata T, Okada Y. A role of reactive oxygen species in apoptotic activation of volume-sensitive Cl\(^{-}\) channel. Proc Natl Acad Sci USA 2004; 101:6770-3; PMID:15096609; http://dx.doi.org/10.1073/pnas.0401604101
[4] Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S. Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J Physiol 2001; 532:3-16; PMID:11283221; http://dx.doi.org/10.1111/j.1469-7793.2001.0003g.x
[5] Hoffmann EK, Lambert IH, Pedersen SF. Physiology of cell volume regulation in vertebrates. Physiol Rev 2009; 89:193-277; PMID:19126758; http://dx.doi.org/10.1152/physrev.00037.2007
[6] Okada Y. Volume expansion-sensing outward-rectifier Cl\(^{-}\) channel: fresh start to the molecular identity and volume sensor. Am J Physiol Cell Physiol 1997; 273:C755-89
[7] Qiu Z, Dubin AE, Mathur J, Tu B, Reddy K, Miraglia LJ, Reinhardt J, Orth AP, Patapoutian A. SWELL1, a plasma membrane protein, is an essential component of volume-regulated anion channel. Cell 2014; 157:447-458; PMID:24725410; http://dx.doi.org/10.1016/j.cell.2014.03.024
[8] Voss FK, Ullrich F, Munch J, Lazarow K, Lutter D, Mah N, Andrade-Navarro MA, von Kries JP, Staubert T, Jentsch TJ. Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC. Science 2014; 344:634-8; PMID:24790029; http://dx.doi.org/10.1126/science.1252826
[9] Syeda R, Qiu Z, Dubin AE, Murthy SE, Florendo MN, Mason DE, Mathur J, Cahalan SM, Peters EC, Montal M, et al. LRRC8 proteins form volume-regulated anion channels that sense ionic strength. Cell 2016; 164:499-511; PMID:26824658; http://dx.doi.org/10.1016/j.cell.2015.12.031
[10] Ullrich F, Reinecke SM, Voss FK, Staubert T, Jentsch TJ. Inactivation and anion selectivity of volume-regulated VRAC channels depend on carboxy-terminal residues of the first extracellular loop. J Biol Chem 2016; 291:17040-8; PMID:27325695
[11] Sato-Numata K, Numata T, Inoue R, Okada Y. Distinct pharmacological and molecular properties of the acid-sensitive outwardly rectifying (ASOR) anion channel from those of the volume-sensitive outwardly rectifying (YSOR) anion channel. Pflügers Arch Eur J Physiol 2016; 468:795-803; http://dx.doi.org/10.1007/s00424-015-1786-1
[12] Auzanneau C, Thoreau V, Kitzi A, Becq F. A Novel voltage-dependent chloride current activated by extracellular acidic pH in cultured rat Sertoli cells. J Biol Chem 2003; 278:19230-6; PMID:12637509; http://dx.doi.org/10.1074/jbc.M301096200
[13] Nobels M, Higgins CF, Sardini A. Extracellular acidification elicits a chloride current that shares characteristics with ICl(swell). Am J Physiol Cell Physiol 2004; 287:C1426-35; PMID:15306547; http://dx.doi.org/10.1152/ajpcell.00549.2002
[14] Sato-Numata K, Numata T, Okada T, Okada Y. Acid-sensitive outwardly rectifying (ASOR) anion channels in human epithelial cells are highly sensitive to temperature and independent of ClC-3. Pflügers Arch Eur J Physiol 2013; 465:1535-43; http://dx.doi.org/10.1007/s00424-013-1296-y
[15] Sato-Numata K, Numata T, Okada Y. Temperature sensitivity of acid-sensitive outwardly rectifying (ASOR) anion channels in cortical neurons is involved in hypothermic neuroprotection against acidotoxic necrosis. Channels (Austin) 2014; 8:278-83; PMID:24476793; http://dx.doi.org/10.4161/chan.27748
[16] Wang HY, Shimizu T, Numata T, Okada Y. Role of acid-sensitive outwardly rectifying anion channels in acidosis-induced cell death in human epithelial cells. Pflügers Arch Eur J Physiol 2007; 454:223-33; http://dx.doi.org/10.1007/s00424-006-0193-z
[17] Lambert S, Oberwinkler J. Characterization of a proton-activated, outwardly rectifying anion channel. J Physiol 2005; 567:191-213; PMID:15961423; http://dx.doi.org/10.1113/jphysiol.2005.089888
[18] Pedersen SF, Klausen TK, Nilius B. The identification of a volume-regulated anion channel: an amazing Odyssey. Acta Physiol (Oxf) 2015; 213:686-81; PMID:25565132; http://dx.doi.org/10.1111/apha.12450
[19] Pedersen SF, Okada Y, Nilius B. Biophysics and physiology of the volume-regulated anion channel (VRAC)/volume-sensitive outwardly rectifying anion channel (VSOR). Pflügers Arch Eur J Physiol 2016; 468:371-83; http://dx.doi.org/10.1007/s00424-015-1781-6
[20] Okada T, Sato-Numata K, Islam MR, Sabirov RZ, Okada Y. Examination of the role of LRRC8A in the function of volume-sensitive outwardly rectifying anion channel (VSOR). J Physiol Sci 2016; 65 (Suppl 1): S120
[21] Abascal F, Zardoya R. LRRC8 proteins share a common ancestor with pannexins, and may form hexameric channels involved in cell-cell communication. Bioessays 2012; 34:551-60; PMID:22532330; http://dx.doi.org/10.1002/bies.201100173