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LRRC8 proteins form volume-regulated anion channels that sense ionic strength

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Summary

The volume-regulated anion channel (VRAC) is activated when a cell swells, and plays a central role in maintaining cell volume in response to osmotic challenges. SWELL1 (LRRC8A) was recently identified as an essential component of VRAC. However, the identity of the pore-forming subunits of VRAC, and how the channel is gated by cell swelling are unknown. Here we show that SWELL1 with up to four other LRRC8 subunits assemble into heterogeneous complexes of ~800 kDa. When reconstituted into bilayers, LRRC8 complexes are sufficient to form anion channels activated by osmolality gradients. In bilayers as well as in cells, the single-channel conductance of the complexes depends on the LRRC8 composition. Finally, low ionic strength (Γ), in the absence of an osmotic gradient, activates the complexes in bilayers. These data demonstrate that LRRC8 proteins together constitute the VRAC pore, and that hypotonic stress can activate VRAC through a decrease in cytoplasmic Γ.

Introduction

The ability to maintain constant cell volume is fundamental to cell function. Osmotically swollen cells restore their original volume and protect themselves via a process called regulatory volume decrease (RVD). This tightly controlled RVD is achieved by the

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Author Contributions: R.S. designed, performed and analyzed the experiments related to reconstitution of protein in lipid bilayers. Z.Q. initiated and lead the study, performed all aspects of biochemistry with help from M.N.F. Z.Q., M.N.F., and S.M.C. generated knockout cell lines. A.E.D. co-initiated the study, contributed to research strategy, conducted whole-cell and cell-attached patch experiments. S.E.M. performed and analyzed single-channel experiments in cells. D.E.M. and E.C.P. performed mass spectrometry analysis, J.M. provided molecular cloning support, and M.M. provided lab resources and critical insight into the manuscript. R.S., Z.Q., A.E.D. and A.P. wrote the manuscript.

activation of swelling-activated ion channels and K^+-Cl^- co-transporters mediating ion and osmolyte efflux together with osmotically obligated water. Swelling-activated chloride currents ($I_{Cl,swell}$), mediated by VRAC, have been observed in nearly every vertebrate cell type including lymphocytes, neurons, astrocytes, cardiac myocytes and endothelial cells (Akita and Okada, 2014; Hoffmann et al., 2009; Pedersen et al., 2015).

LRRC8A (also named “SWELL1”) was recently shown to be an essential component of VRAC since knockdown or deletion of SWELL1 abolished $I_{Cl,swell}$ (Qiu et al., 2014; Voss et al., 2014). SWELL1 (LRRC8A) is conserved across vertebrate species and four additional homologous family members (LRRC8B-E) are usually present in their genomes (Abascal and Zardoya, 2012; Kubota et al., 2004). Deletion and rescue studies have shown that VRAC requires SWELL1 and at least one other LRRC8 subunit for function (Voss et al., 2014). Co-expression of LRRC8 subunits in heterologous systems has revealed subunit interactions by co-immunoprecipitation (Lee et al., 2014; Voss et al., 2014). A scanning cysteine mutagenesis approach identified the residue threonine 44 (T44) just external to the predicted transmembrane domain 1 of SWELL1, which when mutated altered the relative preference for two anions (I^- and Cl^-) suggesting SWELL1 is at a minimum in close proximity of the pore (Qiu et al., 2014). The extent of VRAC inactivation at positive voltages was subunit dependent suggesting LRRC8 proteins are likely associated with the channel (Voss et al., 2014). A recent report revealing subunit specific effects on taurine flux is consistent with LRRC8C-E contributing to the pore (Planells-Cases et al., 2015). These studies indicate that VRAC is composed of at least two subunits that are part of an anion selective channel. However, SWELL1 overexpression caused decreased $I_{Cl,swell}$ (Qiu et al., 2014; Voss et al., 2014), and LRRC8 members together with SWELL1 could not increase $I_{Cl,swell}$ above wildtype (WT) levels, suggesting that they may not be sufficient for VRAC activity. This raises questions on whether SWELL1/LRRC8s are pore forming subunits, and hints at the requirement for other limiting factors/proteins (Kunzelmann, 2015; Qiu et al., 2014; Voss et al., 2014).

The mechanisms by which VRAC is activated by cell swelling have been debated for decades. Water influx and subsequent swelling has multiple consequences that involve local decreases in ion concentrations and changes in macromolecule crowding (Zhou et al., 2008), alterations in plasma membrane domains (Trouet et al., 1999), potential changes in bilayer tension (Anishkin et al., 2014; Brohawn et al., 2014), alterations in cytoskeleton and activation of second messenger pathways including kinases and phosphatases, among others (Hoffmann et al., 2009; Pedersen et al., 2015). Importantly, it is unknown whether VRAC itself contains a sensor for the adequate stimulus during swelling, or if gating requires other cellular components (e.g., cytoskeleton, enzymes, and second messengers). The most discussed mechanisms are increased membrane stress and disruption of cytoskeleton (Hoffmann et al., 2009; Nilius et al., 1996). Very few studies have addressed whether local changes in the ionic environment can stimulate gating (Cannon et al., 1998; Nilius et al., 1998). In the present study we address two critical unsolved questions: what are the proteins that form the VRAC channel pore, and how does cell swelling lead to VRAC activation?

Results

Biochemical analysis of SWELL1-containing protein complexes

To biochemically characterize SWELL1 (LRRC8A) and its associated proteins we generated a HeLa cell line that could be induced to express SWELL1 tagged with FLAG at its carboxy-terminus (SWELL1-FLAG) (Figures 1A **and** S1). SWELL1-FLAG rescued swelling-activated chloride currents ($I_{Cl,swell}$) in SWELL1 knockdown cells just like the WT gene (data not shown), suggesting the tag has no obvious effect on SWELL1 function. Consistent with previous reports (Voss et al., 2014; Qiu et al., 2014), SWELL1 overexpression induced by high tetracycline (TET) concentrations (≥50 ng/ml) decreased $I_{Cl,swell}$ (Figure S1B). This inhibitory effect was not observed at low TET concentrations (5 and 10 ng/ml), which only increased SWELL1 mRNA expression within two-fold above its endogenous level. We utilized 5 ng/ml TET for our biochemical experiments to ensure that ratios of SWELL1 and associated proteins were not considerably disrupted (uninduced cells expressed insufficient amounts of SWELL1-FLAG for biochemical studies, Figures 1A–1C). FLAG affinity purification from lysates of cells treated with 5 ng/ml TET revealed only one prominent protein band at ~800 kDa after native gel electrophoresis and Coomassie blue staining (Figure 1B). Western blots with an anti-SWELL1 antibody confirmed that this band contained SWELL1 (Figure 1C). To determine whether additional proteins might loosely interact with the ~800 kDa complex, we treated live cells with the cross-linker paraformaldehyde (PFA) before the purification procedure and found little or no detectable increase in the size of the SWELL1 protein complex on the native gel (Figures 1D vs. 1B–1C).

LRRC8B, LRRC8C, LRRC8D or LRRC8E each co-immunoprecipitates with SWELL1 in a heterologous overexpression system (Voss et al 2014). To identify the proteins associated with SWELL1 in native complexes, we subjected the purified ~800 kDa band to mass spectrometry analysis and found that in addition to SWELL1, many peptides derived from all four LRRC8 family members were detected (Figure 1E). No other specific interacting proteins were consistently observed, including known pore-forming ion channels (Table S1). Furthermore, mass spectrometry of the entire purified solution without gel separation revealed similar results (data not shown). These data indicate that although it is possible SWELL1 associates with other proteins in addition to its LRRC8 homologs, such an association might not be strong enough to withstand the FLAG affinity purification process. We refer to SWELL1 and its associated LRRC8 homologs as “SWELL1-containing complexes”.

We next used the cross-linker formaldehyde (FA) to examine the oligomeric state of the subunits. FA-treated samples subjected to denaturing electrophoresis revealed at least five major bands in addition to larger unresolved molecular species (Figure 1F). The distribution of bands (consistent with increments of ~95 kDa, the predicted size of each subunit) suggests that they correspond to monomer, dimer, trimer, tetramer, pentamer and potentially higher-order oligomers. LRRC8 proteins are proposed to form hexamers based on their homology with pannexins (Abascal and Zardoya, 2012). The ~800 kDa complex observed on native gels (Figures 1B–1D) would predict up to eight subunits per complex if only the

primary sequence is considered. However, slow migration and overestimation of molecular masses are not uncommon for membrane protein complexes due to many factors, such as glycosylation (Voss et al., 2014) and bound detergent and lipid molecules which can add up to 20% mass (Wittig et al., 2010). Considering the potential size overestimation, the actual mass of SWELL1-containing complexes may vary roughly between six to eight times the predicted molecular weight of single LRRC8 polypeptides, suggesting the presence of six to eight LRRC8 subunits per complex.

In an effort to address the stoichiometry of LRRC8 proteins in the purified complex we used quantitative mass spectrometry with stable-isotope-labeled peptide standards (Table S2). Surprisingly, the relative concentration varied dramatically among each LRRC8 protein (Figure 1E), with SWELL1 being the most abundant, and LRRC8B, the most rare. These data are consistent with the lack of a specific immutable combination of subunits in each complex. Thus, purified complexes appear to be a heterogeneous assortment of multimers composed of mainly SWELL1 in combination with other LRRC8 subunits.

Functional reconstitution of SWELL1-containing complexes in lipid bilayers

To assess whether purified LRRC8 protein complexes can form functional channels consistent with mediating $I_{Cl,swell}$, we used a simple bilayer system amenable to challenge with osmotic stimuli. Protein was supplemented in the droplet attached to the commanding potential electrode termed *cis*, in the presence of ATP to block channels that may insert into the bilayer with extracellular domains exposed to the *cis* side (Jackson and Strange, 1995; Tsumura et al., 1996); the *trans* droplet was grounded. A hypotonic gradient was generated by supplementing 300 mM mannitol (*cis*) to cause water influx into this droplet (i.e. cytoplasmic side). Although we initially chose 500 mM KCl to enhance signal-to-noise and to resolve detailed features of single-channel currents, we found similar results using physiological osmolarities and salt concentrations (see below). Channel activity was not observed when the protein was reconstituted in isotonic solution (Figure 1G), whereas a spectrum of single-channel conductance (γ) ranging from ~10 pS to 50 pS at −100 mV and open probability (P_o) ranging from 0.1 to 0.6 were reproducibly observed when complexes were exposed to a hypotonic stimulus (examples in Figures 1H–1J). Importantly, activity was observed only when mannitol was introduced asymmetrically in the *cis* droplet (Figures 1H–1J) but not when equal concentrations of mannitol were present in both *cis* and *trans* droplets (Figure S2A). To exclude the possibility that mannitol elicited activity by a mechanism distinct from osmotic disequilibrium, we tested whether sucrose could also stimulate activity. Distinct channel activity with a range of γ was observed when the hypotonic stimulus was generated by a sucrose gradient, indicating that mannitol is not specifically activating channel complexes (Figures S2B–S2C). To assess whether ATP is required for channel function, we performed reconstitution studies in the absence of ATP in both *cis* and *trans* droplets, while maintaining the relevant hypotonic stimulus. ATP was not required to observe distinct channel activity (Figure S3A), however, our bilayer studies cannot address whether there is a higher propensity for inactive channels in the absence of ATP. The hypotonicity-induced channel activity was blocked by injection of a VRAC blocker 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1*H*-inden-5-yl)oxy]butanoic acid (DCPIB) in either droplet (Figure 1K).

To test whether the reconstituted channels retained ion selectivity, lipid bilayers were created with 500 mM potassium gluconate on both sides (Figure S3B). Hypotonicity-induced currents at −100 mV in 500 mM potassium gluconate were smaller in amplitude (ranging from ~0.4 to 0.8 pA) than those in 500 mM KCl solution (~1–5 pA), indicating the dependence of currents on the anion species (not K^+) and that channels are capable of fluxing gluconate to some extent. These experiments provide evidence that isolated SWELL1-containing complexes incorporated into lipid bilayers act as *bonafide* anionic channels induced by hypotonicity and sensitive to DCPIB.

To address the specific role of various LRRC8 subunits in SWELL1-containing complexes, we constructed cell lines from which specific combinations of subunits can be purified. We focused on heteromers containing SWELL1 and only one other LRRC8 subunit. Specifically, we used clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology to disrupt multiple *LRRC8* genes in HeLa inducible cells (Table S3). The purified SWELL1-containing complexes from triple and quadruple knockout (KO) cells migrated to the same position on a native gel as the sample from WT HeLa inducible cells (Figure 2A). This indicates that the oligomeric state of SWELL1-containing complexes remains constant despite the absence of three or all four LRRC8 homologs. Mass spectrometry confirmed the predicted compositions of SWELL1-containing complexes purified from the various KO cell lines (Figure 2B). For example, the protein sample purified from *LRRC8(B/D/E)*$^{-/-}$ cells contained only SWELL1 and LRRC8C (red bar). Next, we characterized $I_{Cl,swell}$ in KO cell lines (Figure 2C). Interestingly, whole-cell current densities in *LRRC8(B/C/E)*$^{-/-}$ and *LRRC8(B/C/D)*$^{-/-}$ cells were similar to WT cells; while *LRRC8(B/D/E)*$^{-/-}$ current densities were reduced by about half. In agreement with previous findings for human HCT116 cells (Voss et al., 2014), $I_{Cl,swell}$ was essentially not detected from *LRRC8(B/C/D/E)* $^{-/-}$cells (Figure 2C) although homomeric SWELL1 complexes ~800 kDa were observed (Figure 2A). These data confirm that SWELL1 and at least one other LRRC8 subunit are required for whole-cell VRAC activity.

A wide range of swelling-induced outwardly rectifying single-channel Cl^- conductances all having the essential characteristics of VRAC have been reported in cells (Nilius et al., 1996; Okada, 1997). In our droplet lipid bilayer experiments, SWELL1-containing complexes purified from WT cells also exhibited a broad γ range at both positive and negative voltages (Figures 2D and 1H–1J). To test whether γ is determined by LRRC8 complex composition, we recorded single-channel currents of purified protein from KO cells expressing only two subunits. Remarkably, proteins purified from *LRRC8(B/D/E)*$^{-/-}$, *LRRC8(B/C/E)*$^{-/-}$ and *LRRC8(B/C/D)*$^{-/-}$ cells produced hypotonicity-induced channel activities with a more limited γ range compared to WT (Figures 2E–2G). For simplicity, we refer to protein complexes purified from *LRRC8(B/D/E)*$^{-/-}$, *LRRC8(B/C/E)*$^{-/-}$ and *LRRC8(B/C/D)*$^{-/-}$ cells as LRRC8 A+C, A+D and A+E, respectively. The γ of LRRC8 A+C (red; Figure 2E), A+D (blue; Figure 2F) and A+E (green; Figure 2G) were calculated at both positive and negative voltages. The calculated γ values of LRRC8 A+C, A+D and A+E differ significantly from each other and were all represented within the conductance spectrum observed for WT. These experiments provide evidence that each LRRC8 heteromeric complex exhibits a

distinct γ range and that the WT sample contains mixtures of heteromers that produces the observed broad γ range.

Surprisingly, although $I_{Cl,swell}$ was not observed in *LRRC8(B/C/D/E)*$^{-/-}$ cells using the standard whole-cell voltage ramp protocol, SWELL1-containing complexes purified from these cells produced DCPIB-sensitive hypotonicity-induced currents at −100 but not +100 mV in lipid bilayers (Figures 2H and S4). The discrepancy between whole-cell and lipid bilayer current recordings for SWELL1 homomers is not currently understood. Technical limitations may prohibit the study of homomers at +100 mV since only steady state current activity is measured in the lipid bilayers. Based on the results from triple and quadruple KO samples, we assign the populations with smallest γ at −100 mV to SWELL1 homomers (brown; 8–16 pS) (Figure 2H) that significantly differs from the γ range of heteromeric LRRC8 A+C (35–47 pS), A+D (14–19 pS) and A+E (23–33 pS) complexes at −100 mV (Figures 2E–2G). These results suggest that SWELL1 homomers form stable channels in lipid bilayers (Figure 2H) and co-exist with heteromers composed of SWELL1 and other LRRC8 subunits (Figures 2D–2G). Importantly, channel activity recorded from the purified proteins indicates that the γ range is dependent on the identity of the LRRC8 subunit associating with SWELL1 in the complex. These experiments also rule out the possibility that a rare impurity, rather than SWELL1-containing complex, accounts for the channel activities we observe.

LRRC8-mediated single-channel currents from swollen HeLa cells

Next, we validated the VRAC γ variability in native cells. We performed cell-attached patch clamp recordings from HeLa cells in isotonic (ISO) vs. hypotonic (HYPO; 265 mOsm/Kg) solution to identify VRAC-like single-channel events. Cells were bathed in high K^+ solutions to suppress RVD. We generated another panel of LRRC8 KO lines in WT HeLa cells (Table S4) and used *LRRC8(A/B/C/D/E)*$^{-/-}$ cells which had essentially no $I_{Cl,swell}$ to confirm that any observed activity was indeed mediated by VRAC (data not shown). In HYPO solution, small single-channel currents were observed in ~50% of the *LRRC8(A/B/C/D/E)*$^{-/-}$ patches at positive potentials (resting potential (RP) +100 mV) (1.0 ± 0.07 pA, mean ± SEM, n=20; Figure S5A). Similar activity was also observed when the cells were bathed in ISO solution (1.4 ± 0.3 pA, n=5). These small conductance channels (~10 pS) were also observed in WT cells and had a similar incidence of occurrence, independent of swelling (in ISO: 0.98 ± 0.04 pA, n=5; in HYPO: 1.0 ± 0.1 pA, n=10, Figure S5B). Since these single-channel currents were independent of cell type and osmotic stimulus, any single-channel activity with amplitudes less than 1.7 pA (two standard deviations above the mean) was deemed as background. In addition to this background current, when WT cells were bathed in HYPO solution and cell-attached patches from swollen cells were made, larger amplitude, inactivating single-channel currents were observed at positive potentials. Importantly, these larger currents were not observed in *LRRC8(A/B/C/D/E)*$^{-/-}$ control cells. Single-channel currents from swollen WT HeLa cells revealed a pronounced heterogeneity in current amplitude from 1.8 pA to 7.3 pA at +100 mV above RP (Figure 3A). These types of currents were not observed in patches from WT cells in ISO solution (n=5). To determine whether conductance depended on subunit composition, we tested *LRRC8(B/D/E)*$^{-/-}$ and *LRRC8(B/C/E)*$^{-/-}$ cells (Figures 3B–3C).

The corresponding LRRC8 A+C and A+D heteromers exhibited the largest and smallest conductances, respectively, in bilayers. Interestingly, *LRRC8(B/D/E)*$^{-/-}$ cells revealed single-channel currents with amplitudes from 3.1 to 6.4 pA (4.65 ± 0.18 pA, n=22; Figure 3B) while *LRRC8(B/C/E)*$^{-/-}$ showed single-channel currents from 2.0 to 3.4 pA (2.48 ± 0.18 pA, n=7; Figure 3C), a statistically significant difference (Student's *t*-test, P<0.001). Currents greater than 1.7 pA were not observed when cells were bathed in ISO solution (n=12 and 10 for *LRRC8(B/D/E)*$^{-/-}$ and *LRRC8(B/C/E)*$^{-/-}$, respectively). Therefore, the cellular data corroborate the heterogeneity observed for reconstituted SWELL1-containing complexes in droplet lipid bilayers and underscore the importance of subunit composition to channel conductance.

LRRC8 subunit composition dictates VRAC rectification

One of the characteristic features of VRAC is its outward rectification, i.e. larger outward currents (Cl^- influx) than inward currents (Cl^- efflux) at positive vs. negative membrane potentials, respectively (Nilius et al., 1996; Pedersen et al., 2015). Single-channel analysis and all-points current histograms revealed that purified LRRC8 A+C, A+D and A+E heteromers retained outward rectification in lipid bilayers (Figures 4A–4C). The currents at positive voltage were larger compared to currents at negative voltage for all complexes (Figures 4A–4C **and** S6). To determine whether rectification properties depended on subunit composition, we analyzed the extent of single-channel rectification both for γ (Figure 4D–4F) and P_o (Figure 4G–4I). The ratio of γ observed at positive to negative potentials was the same for LRRC8 A+C, A+D and A+E, suggesting that the extent of rectification due to γ is the same for all complexes tested (Figure 4F). Interestingly, the ratio of P_o acquired at +100 to that of −100 mV was significantly greater for LRRC8 A+D compared to LRRC8 A+C and LRRC8 A+E (Figure 4I). Remarkably, the outward rectification of $I_{Cl,swell}$ in whole-cell recordings of the triple KO cells mirrored that observed in lipid bilayers (Figures 4J–4K). The ratio of currents ($I_{outward}/I_{inward}$) recorded from *LRRC8(B/C/E)*$^{-/-}$ cells was larger compared to *LRRC8(B/D/E)*$^{-/-}$ and *LRRC8(B/C/D)*$^{-/-}$ cells (Figure 4K). Our single-channel data confirm previous studies that whole-cell rectification is in part due to differences in γ at positive vs. negative potentials (Nilius et al., 1996). Bilayer studies suggest that P_o can also contribute to rectification in LRRC8D-containing heteromeric complexes. The inability to record for longer times from cell-attached patches on swollen cells at large positive potentials limited us from obtaining substantial data to determine accurate P_o values and therefore prohibited a direct comparison between bilayers and cells. Nonetheless, rectification properties depend on LRRC8 subunit composition.

LRRC8 subunits associating with SWELL1 contribute to ion selectivity

Since it is generally agreed that channel conductance is a property of the pore, these bilayer data indicate that LRRC8C-E can contribute to pore properties of SWELL1-containing complexes. We next examined whether LRRC8 subunit composition alters the anion permeation properties of heteromeric complexes in cells, another pore-related property. Our previous mutagenesis screen identified T44 as a critical residue of SWELL1, as cysteine replacement of this residue (T44C) significantly increased I^- vs. Cl^- permeability (P_I/P_{Cl}). T44 is predicted to be located at the external boundary of TM1 and, intriguingly, is a conserved residue in the other LRRC8 subunits except LRRC8B. To examine the effect of

the same point mutation in LRRC8C-E subunits, we rescued $I_{Cl,swell}$ in *LRRC8(B/C/D/E)*$^{-/-}$ cells by transfecting either WT or T44C mutant LRRC8 cDNAs. The reversal potential (V_{rev}) of hypotonicity-induced currents was determined when I^- or Cl^- was the only permeant extracellular anion and P_I/P_{Cl} calculated from the difference in V_{rev} as previously described (Qiu et al., 2014). While $I_{Cl,swell}$ in all cells reversed near E_{Cl} as expected, V_{rev} in the I^- solution for all T44C mutants was significantly shifted to more negative potentials compared to their corresponding WT controls (Figures 5A–5B). This indicates that, like SWELL1 T44C, cysteine mutations at T44 in other LRRC8 subunits also enhance P_I/P_{Cl} (Figure 5C). Additionally, compared to native $I_{Cl,swell}$ in HeLa inducible cells, VRAC currents rescued by WT LRRC8C or LRRC8E showed a significantly higher I^- selectivity while WT LRRC8D revealed a reduced P_I/P_{Cl} (Figure 5). This suggests that LRRC8 subunits also affect VRAC I^- vs. Cl^- selectivity in HeLa cells. Taken together, the γ and rectification properties in lipid bilayers and the finding that LRRC8 subunits and their point mutations contribute to anion selectivity demonstrate that LRRC8C-E proteins can contribute to the VRAC pore.

LRRC8 protein complexes are activated by low intracellular ionic strength

Permeation properties of $I_{Cl,swell}$ have been well studied (Akita and Okada, 2014; Pedersen et al., 2015) but the activation mechanism of VRAC at the molecular level is not understood. Hypotonicity-induced cell swelling and increase in the cell volume could evoke numerous physiologically relevant effects including (i) mechanical changes in the cells, (ii) alterations in cellular signaling, and (iii) local decreases in the intracellular ion concentration due to water influx. Our protein purification and reconstitution approach excluded an essential role of cellular components and signaling pathways in the activation of SWELL1-containing complexes. Thus we examined whether any of the remaining mechanisms, i.e. mechanical changes in the membrane or decreases in ionic strength (Γ), are essential for the activity of purified protein complexes in droplet lipid bilayers. To assess the activation mechanism based on volume increase and membrane stretch, LRRC8 A+C, A+D and A+E heteromers were reconstituted in lipid bilayers under ISO conditions (500 mM KCl, 10 mM HEPES, pH 7.4) and 40 nL of this solution was injected into either the *cis* or *trans* droplet of the pre-formed bilayers. This injection resulted in ~20% increase in the droplet volume but did not evoke channel activity for any of the LRRC8 heteromers tested (n=9, data not shown). This stimulus was, however, sufficient to activate mechanically-activated MscS channels (data not shown) (Battle et al., 2009). This suggests that the increase in droplet volume and the subsequent mechanical changes in the lipid bilayer is not the likely cause of channel activity.

Next we screened the effect of low Γ on the activation of SWELL1-containing complexes. Three different salt concentrations were tested in the lipid bilayers: 500 mM KCl to represent high Γ (standard solution for droplet bilayer recordings), 150 mM KCl to represent physiological Γ, and 70 mM KCl to represent low Γ. Channel activity was not observed when LRRC8 A+C, A+D and A+E heteromers were reconstituted in droplet lipid bilayers established from *cis* and *trans* droplets containing 500 (Figure 6A) or 150 mM KCl (Figure 6B, examples shown for LRRC8 A+E). However, robust currents exhibiting $\gamma = 8 \pm 2$ pS (Figure 6C) were recorded from reconstituted LRRC8 A+E heteromers in symmetric 70 mM KCl. Since decreasing the Γ from physiological 150 mM KCl to low 70 mM KCl also

decreases osmolality of the solution from ~300 mOsm/Kg to ~140 mOsm/Kg, we tested whether channel activity in 70 mM KCl was due to decreased Γ or decreased osmolality. LRRC8 A+E activity was still observed when osmolality was kept at physiological levels by supplementing 160 mM mannitol, while decreasing Γ to the 70 mM KCl (Figure 6D) indicating that the protein complex can be activated by lowering the Γ in the absence of an osmotic gradient. Discrete single-channel currents were recorded that exhibited $\gamma = 8 \pm 2$ pS comparable to channel properties observed in the isotonic 70 mM KCl solution (Figure 6C–6D). LRRC8 A+C and A+D activity was also reproducibly observed in isotonic 70 mM KCl (Figure 6E-6F) but not in 150 or 500 mM KCl (data not shown). Regardless of the combination of LRRC8 heteromers, all SWELL1-containing complexes tested produced DCPIB-sensitive currents in droplet bilayers under low Γ conditions (data not shown). These data indicate that SWELL1-containing complexes are activated by lowering the Γ in a minimalistic bilayer system.

Discussion

VRAC is activated during shifts in osmotic homeostasis that lead to cell swelling and contributes to a variety of physiological and pathophysiological processes (Akita and Okada, 2014; Hoffmann et al., 2009; Kunzelmann, 2015). Here we take advantage of our recent identification of SWELL1 (Qiu et al., 2014) as an essential component of VRAC to purify and characterize SWELL1-containing complexes. We gained three fundamental insights using a combination of biochemical and electrophysiological methods. First, VRAC is a heterogeneous collection of high molecular weight (~800 kDa) complexes composed of LRRC8 proteins which, when purified and incorporated in lipid bilayers, can be directly activated by an osmotic gradient. Second, SWELL1 and LRRC8C-E subunits together are sufficient to make pore-forming ion channels; channel conductance, permeability and rectification depend on the nature of the associating non-SWELL1 LRRC8 protein. Third, low Γ directly gates VRAC in a minimalistic bilayer system, giving us a mechanistic insight on how VRAC is gated in response to cell swelling. This finding also indicates that VRAC opening does not require other cellular components, and that the sensor is encoded within the channel complex.

VRAC is a heterogeneous collection of ion channels composed of LRRC8 proteins

By immunoprecipitating SWELL1 from HeLa cells, we identified all other members of LRRC8 family (LRRC8B-E), but no other proteins, specifically associating with SWELL1 in an ~800 kDa native complex. Interestingly, the ratio of associating proteins was not consistent with the existence of a single entity. However, the number of subunits per pore-forming complex appears to be constant irrespective of subunit composition. Given the homology of LRRC8 proteins to pannexin (Abascal and Zardoya, 2012), their predicted unmodified molecular weights (~95 kDa) and their likelihood of being glycosylated (Voss et al., 2014), LRRC8 subunits probably assemble as hexameric channels. SWELL1 is an obligatory subunit in this complex (Qiu et al., 2014; Voss et al., 2014) and at least one of the other four LRRC8 homologs is required (Figure 2 and (Voss et al., 2014)). Furthermore, the exact stoichiometry and intramolecular arrangement of SWELL1 and other LRRC8 subunits within the pore-forming complex is still unknown, and the anticipated flexibility in

stoichiometry and arrangement suggests the existence of a very large cohort of disparate VRAC channels. Given the complexity, future single-molecule and structural studies are required to determine the stoichiometry and oligomeric state of LRRC8 heteromers. Several ligand-gated ion channel families exhibit similar flexibilities in subunit composition and their arrangement (Hille, 2001). However, this structural diversity with its consequential functional diversity in channel biophysical properties makes VRAC unique among ion channels.

Specific LRRC8 subunits associate with SWELL1 to dictate channel properties

A range of intermediate γ for VRAC has been described among different tissues and cells over the last three decades (Nilius et al., 1996; Okada, 1997). Although the currents were swelling-dependent, the early studies suffered from a lack of molecular identity of VRAC and specific pharmacological tools. Furthermore, the underlying causes of this heterogeneity were not known.

The identification of the LRRC8 gene family as essential components of VRAC enabled us with certainty to identify LRRC8-dependent swelling-induced single-channel currents in cellular assays. Cell-attached patches from swollen HeLa cells revealed a wide range of current amplitudes. Although most early studies using cell-attached patches did not comment on current amplitude heterogeneity (Okada et al., 1994; Weiss and Lang, 1992), one study reported channels with at least two significantly different conductances with features of $I_{Cl,swell}$ (e.g., run down, inactivation at positive voltages) (Solc and Wine, 1991). Our single-channel studies conducted in droplet lipid bilayers also demonstrated the conductance heterogeneity of LRRC8 proteins. Outward single-channel currents in WT HeLa cells revealed a wide range of γ from 18 to 73 pS at +100 mV (assuming a RP of 0 mV). However, when cells expressed only two of the five subunits, *LRRC8(B/D/E)*$^{-/-}$ and *LRRC8(B/C/E)*$^{-/-}$, the γ range became narrow and non-overlapping, similar to those observed in the lipid bilayers. The γ values obtained from swollen HeLa cells recorded in ~100 mM Cl^- lie between those observed in lipid bilayers formed in the presence of either 70 mM or 500 mM Cl^-. Regardless of the assay, the conductance of purified complexes reconstituted into lipid bilayers and VRAC expressed in cellular membranes depend on the identity of the associating subunit (LRRC8C-E). Consistent with previous reports (Nilius et al., 1996; Okada, 1997), single-channel currents from all combinations tested revealed similar rectification where outward is greater than inward current in both cellular and lipid bilayer assays.

Another way to probe whether LRRC8-associating proteins contribute to pore properties is to determine whether mutations alter relative permeability. Mutating the homologous T44 residue in LRRC8C, LRRC8D, and LRRC8E to a cysteine increased the relative permeability of I^- compared to Cl^-, similar to that observed with SWELL1 (Qiu et al., 2014). Interestingly, WT LRRC8D subunits had significantly lower I^- vs. Cl^- permeability than either LRRC8C or LRR8CE. These results indicate that not only SWELL1 but each LRRC8 protein contributes to the permeation properties of VRAC.

The heterogeneity of functional VRAC properties we observed in bilayers must be a consequence of the diversity of heteromeric channels identified in our biochemical studies.

The combination of specific LRRC8 homologs determines intrinsic channel properties, such as conductance and relative permeability. We propose that the heterogeneity of most, if not all, VRAC biophysical properties between different native tissues is regulated by differential expression of LRRC8 isoforms. Interestingly, another common characteristic of VRAC whole-cell current (i.e., its variable inactivation kinetics at high positive potentials) was also shown to depend on LRRC8 homolog composition (Voss et al., 2014). Recently, LRRC8D, but not LRRC8C or LRRC8E, was shown to be required for the influx of certain chemotherapeutics (Planells-Cases et al., 2015). The physiological relevance of having molecularly diverse VRAC channels is currently unknown but is likely that cell and tissue specific expression of particular LRRC8 proteins is important in specifying roles of VRAC in that tissue. LRRC8C has been reported to contribute to adipocyte differentiation and diet-induced obesity (Hayashi et al., 2011; Tominaga et al., 2004) but whether this is due to its VRAC function is not known. Our results provide the basis to explore these questions through expression analysis and genetic studies of all LRRC8 isoforms.

Volume Sensing Mechanism of LRRC8 channel proteins

The detection of cell volume expansion and subsequent transduction to VRAC activation are poorly understood. We have taken a minimalistic bilayer approach to study VRAC activation in the absence of cellular components that have been proposed to be involved in this process, such as cytoskeleton, caveoli, enzymes and second messenger systems. What we are left with is purified SWELL1-containing LRRC8 complexes and a simple lipid bilayer at the interface between two droplets, which can be experimentally manipulated. We can specifically test effects of mechanically-induced stresses on the membrane or a decrease in “intracellular” Γ resulting from diffusion of water across the membrane. Purified complexes in these bilayers are silent unless an osmotic gradient is imposed between droplets. Under these conditions we found that injection of isotonic solution into one droplet to increase its volume and perturb the bilayer was not sufficient to induce channel activity, although control experiments indicated the method activates MscS channels. On the other hand, when bilayers are formed with lower Γ in the *cis* (i.e., cytoplasmic side) droplet, channels were active. Thus, these purified ion channels gate when the ionic concentration is reduced, indicating that SWELL1-containing complexes encode a sensor for low Γ. This intriguing hypothesis that decreased Γ rather than cell-swelling itself is an adequate stimulus for VRAC was initially proposed by Nilius (Nilius et al., 1998; Sabirov et al., 2000), Strange (Cannon et al., 1998), and their colleagues.

A bacterial transporter OpuA is also triggered directly by an Γ mechanism (Mahmood et al., 2006). In this case, high Γ causes the dissociation of surface-exposed cationic regions from negative phospholipid head-groups (electrostatic switching mechanism) leading to conformational changes in the protein (Biemans-Oldehinkel et al., 2006; Poolman et al., 2004). A similar interaction of cationic residues and negatively charged lipids has been described for the mammalian mechanoactivated TREK-1 potassium channel (Chemin et al., 2005). Normal or high Γ may maintain VRAC in a closed conformation such that influx of water and reduction of ionic concentrations gates the channel. For instance, surface exposed cationic domains, highly charged domains, and salt bridges have been shown to be critical for HCN2 and CNGA1 channel gating (Craven and Zagotta, 2004). The obligatory subunit

SWELL1 may encode the sensor or the sensor may be encoded by each associating subunit or the interfaces they make with SWELL1 or each other. The importance of subunit interfaces to gating is underscored in the ligand-gated ionotropic receptors (Green and Nayeem, 2015). Future mutagenesis and chimeric approaches will enable the identification of the Γ sensor(s) within SWELL1-containing complexes. Predicted cytosolic domains that might be involved in sensing low Γ include the highly cationic TM2-TM3 intracellular loop and proximal C terminal tail as well as negatively charged regions in the case of intramolecular interactions. It should be noted, however, that the present study does not address whether low Γ is the only mechanism to activate $I_{Cl,swell}$ in cells; it is likely that other mechanisms play concerted roles. Indeed, it is known that VRAC can also be activated in the absence of cell swelling (Hoffmann et al., 2009).

In summary, we demonstrate that purified SWELL1-containing complexes with at least one other LRRC8 subunit can form functional ion channels that can be directly activated by physiological stimuli. We show in both minimalistic bilayer systems and HeLa cells that the particular LRRC8 subunit (LRRC8C-E) associating with SWELL1 determines the single-channel conductance and the relative permeability of $I_{Cl,swell}$ for iodide and chloride indicating these family members contribute to the pore. While other pathways such as phosphorylation and cytoskeletal interactions may contribute in intact cells, a sensor for low Γ is an integral part of purified SWELL1-containing complexes. With this new data, the structural requirements for VRAC activation may now be addressed. A more complete understanding of volume-sensing mechanisms will undoubtedly lead to insights into the physiological roles of VRAC and help guide future therapeutic approaches in ischemia, stroke, and other pathologies.

Methods

Electrophysiology

Whole-cell patch clamp recordings were performed as described previously (Qiu et al., 2014) except, for permeability studies, recording pipettes contained (in mM) 130 CsCl, 10 HEPES, 4 MgATP (pH 7.2), LRRC8(B/C/D/E)$^{-/-}$ HeLa Flp-In T-REx cells were transfected with WT or T44C LRRC8C, LRRC8D or LRRC8E and cultured without TET, and the experimenter was blinded to cell type. Cell-attached patch recordings were made on swollen cells using 7–15 MΩ pipettes containing (in mM) 95 N-Methyl-D-Glucamine (NMDG), 4 $MgCl_2$, 4 NaCl, 5 TEA-Cl, 10 HEPES, 5 dextrose, 0.1 $CdCl_2$, and 100 mannitol using a protocol modified from previous studies (Okada et al., 1994; Wang et al., 2005). Cells were bathed in a high extracellular KCl solution (either 265 or 300 mOsm) to depolarize cells toward 0mV and to inhibit RVD (Okada et al., 1994). The 265 mOsm solution contained (in mM): 100 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 dextrose, KOH to pH 7.4 and 265 mOsm/Kg with mannitol (Vapro Vapor Pressure Osmometer, Wescor). The 300 mOsm solution included extra mannitol (35 mM).

Reconstitution of SWELL1-containing complexes in droplet lipid bilayers

Droplet lipid bilayers were formed as described previously (Bayley et al., 2008; Syeda et al., 2008). Briefly, lipid bilayers were formed between two droplets containing in (mM): 0.5

1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), 500 KCl, 20 HEPES, at pH 7.4 in a hexadecane medium. For low Γ experiments 500 mM KCl was replaced by either 150 or 70 mM KCl. Protein was added in the form of proteoliposomes in the *cis* droplet. Only single-channel records were selected for analysis to avoid anomalies in conductance and open probability determination, however multiple channels were also observed.

Isotonic and hypotonic conditions in droplet lipid bilayers

Mannitol (300 mM) was supplemented asymmetrically in the *cis* droplet to generate an osmolality imbalance causing water flux into the *cis* droplet. An isotonic condition refers to the symmetric solution in both droplets (e.g., 500 or 150 or 70 mM KCl, or 70 mM KCl +160 mM mannitol).

Protein orientation in droplet lipid bilayers

We used the ATP-sided block approach as previously described for whole-cell VRAC currents to block inserted channels in the outside-in configuration (Jackson and Strange, 1995). In this method, the *cis* droplet contained Na_2-ATP (4 mM). Activated SWELL1-containing complexes were blocked by adding 4 mM Na_2-ATP to the *trans* droplet, indicating extracellular domains faced the *trans* droplet. Channels usually inserted such that the intracellular side of the protein remained in the *cis* droplet.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Abascal F, Zardoya R. LRRC8 proteins share a common ancestor with pannexins, and may form hexameric channels involved in cell-cell communication. BioEssays. 2012; 34:551–560. [PubMed: 22532330]

Akita T, Okada Y. Characteristics and roles of the volume-sensitive outwardly rectifying (VSOR) anion channel in the central nervous system. Neuroscience. 2014; 275:211–231. [PubMed: 24937753]

Anishkin A, Loukin SH, Teng J, Kung C. Feeling the hidden mechanical forces in lipid bilayer is an original sense. Proc Natl Acad Sci U S A. 2014; 111:7898–7905. [PubMed: 24850861]

Battle AR, Petrov E, Pal P, Martinac B. Rapid and improved reconstitution of bacterial mechanosensitive ion channel proteins MscS and MscL into liposomes using a modified sucrose method. FEBS Lett. 2009; 583:407–412. [PubMed: 19111548]

Bayley H, Cronin B, Heron A, Holden MA, Hwang WL, Syeda R, Thompson J, Wallace M. Droplet interface bilayers. Mol Biosyst. 2008; 4:1191–1208. [PubMed: 19396383]

Biemans-Oldehinkel E, Mahmood NA, Poolman B. A sensor for intracellular ionic strength. Proc Natl Acad Sci U S A. 2006; 103:10624–10629. [PubMed: 16815971]

Brohawn SG, Su Z, MacKinnon R. Mechanosensitivity is mediated directly by the lipid membrane in TRAAK and TREK1 K^+ channels. Proc Natl Acad Sci. 2014; 111:3614–3619. [PubMed: 24550493]

Cannon CL, Basavappa S, Strange K. Intracellular ionic strength regulates the volume sensitivity of a swelling-activated anion channel. Am J Physiol. 1998; 275:C416–C422. [PubMed: 9688595]

Chemin J, Patel AJ, Duprat F, Lauritzen I, Lazdunski M, Honore E. A phospholipid sensor controls mechanogating of the K+ channel TREK-1. EMBO J. 2005; 24:44–53. [PubMed: 15577940]

Craven KB, Zagotta WN. Salt Bridges and Gating in the COOH-terminal Region of HCN2 and CNGA1 Channels. J Gen Physiol. 2004; 124:663–677. [PubMed: 15572346]

Green T, Nayeem N. The multifaceted subunit interfaces of ionotropic glutamate receptors. J Physiol. 2015; 593:73–81. [PubMed: 25556789]

Hayashi T, Nozaki Y, Nishizuka M, Ikawa M, Osada S, Imagawa M. Factor for Adipocyte Differentiation 158 Gene Disruption Prevents the Body Weight Gain and Insulin Resistance Induced by a High-Fat Diet. Biol Pharm Bull. 2011; 34:1257–1263. [PubMed: 21804215]

Hille, B. Ion Channels of Excitable Membranes. Sunderland: Sinauer Associates, Inc; 2001.

Hoffmann EK, Lambert IH, Pedersen SF. Physiology of cell volume regulation in vertebrates. Physiol Rev. 2009; 89:193–277. [PubMed: 19126758]

Jackson PS, Strange K. Characterization of the voltage-dependent properties of a volume-sensitive anion conductance. J Gen Physiol. 1995; 105:661–676. [PubMed: 7544824]

Kubota K, Kim JY, Sawada A, Tokimasa S, Fujisaki H, Matsuda-Hashii Y, Ozono K, Hara J. LRRC8 involved in B cell development belongs to a novel family of leucine-rich repeat proteins. FEBS Lett. 2004; 564:147–152. [PubMed: 15094057]

Kunzelmann K. TMEM16, LRRC8A, bestrophin: chloride channels controlled by Ca(2+) and cell volume. Trends Biochem Sci. 2015; 40:535–543. [PubMed: 26254230]

Lee CC, Freinkman E, Sabatini DM, Ploegh HL. The Protein Synthesis Inhibitor Blasticidin S Enters Mammalian Cells via Leucine-rich Repeat-containing Protein 8D. J Biol Chem. 2014; 289:17124–17131. [PubMed: 24782309]

Mahmood NABN, Biemans-Oldehinkel E, Patzlaff JS, Schuurman-Wolters GK, Poolman B. Ion Specificity and Ionic Strength Dependence of the Osmoregulatory ABC Transporter OpuA. J Biol Chem. 2006; 281:29830–29839. [PubMed: 16844687]

Nilius B, Eggermont J, Voets T, Droogmans G. Volume-activated Cl− channels. Gen Pharmacol. 1996; 27:1131–1140. [PubMed: 8981057]

Nilius B, Prenen J, Voets T, Eggermont J, Droogmans G. Activation of volume-regulated chloride currents by reduction of intracellular ionic strength in bovine endothelial cells. J Physiol. 1998; 506(Pt 2):353–361. [PubMed: 9490864]

Okada Y. Volume expansion-sensing outward-rectifier Cl− channel: fresh start to the molecular identity and volume sensor. Am J Physiol Cell Physiol. 1997; 273:755–789.

Okada Y, Carl Petersen, Machiko K, Shigeru M, Makoto T. Oamotic Swelling Activates Intermediate-conductance Cl− Channels in Human Intestinal Epithelial Cells. Jpn J Physiol. 1994; 44:403–409. [PubMed: 7869602]

Pedersen SF, Klausen TK, Nilius B. The identification of a volume-regulated anion channel: an amazing Odyssey. Acta Physiol (Oxf). 2015; 213:868–881. [PubMed: 25565132]

Planells-Cases R, et al. Subunit composition of VRAC channels determines substrate specificity and cellular resistance to Pt-based anti-cancer drugs. EMBO J. 2015 pii: e201592409.

Poolman B, Spitzer JJ, Wood JM. Bacterial osmosensing: roles of membrane structure and electrostatics in lipid–protein and protein–protein interactions. Biochim Biophys Acta - Biomembr. 2004; 1666:88–104.

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. [PubMed: 24725410]

Sabirov RZ, Prenen J, Tomita T, Droogmans G, Nilius B. Reduction of ionic strength activates single volume-regulated anion channels (VRAC) in endothelial cells. Pflugers Arch. 2000; 439:315–320. [PubMed: 10650983]

Solc C, Wine J. Swelling-induced and depolarization-induced Cl^- channels in normal and cystic fibrosis epithelial cells. Am J Physiol Cell Physiol. 1991; 261:658–674.

Syeda R, Holden MA, Hwang WL, Bayley H. Screening blockers against a potassium channel with a droplet interface bilayer array. J Am Chem Soc. 2008; 130:15543–15548. [PubMed: 18950170]

Tominaga K, Kondo C, Kagata T, Hishida T, Nishizuka M, Imagawa M. The novel gene fad158, having a transmembrane domain and leucine-rich repeat, stimulates adipocyte differentiation. J Biol Chem. 2004; 279:34840–34848. [PubMed: 15184384]

Trouet D, Nilius B, Jacobs A, Remacle C, Droogmans G, Eggermont J. Caveolin-1 modulates the activity of the volume-regulated chloride channel. J Physiol. 1999; 520:113–119. [PubMed: 10517805]

Tsumura T, Oiki S, Ueda S, Okuma M, Okada Y. Sensitivity of Volume-Sensitive Cl− Conductance in Human Cells To Extracellular Nucleotides. Am J Physiol Cell Physiol. 1996; 271:1872–1878.

Voss FK, Ullrich F, Münch J, Lazarow K, Lutter D, Mah N, Andrade-Navarro MA, von Kries JP, Stauber T, Jentsch TJ. Identification of LRRC8 heteromers as an essential component of the volume-regulated anion channel VRAC. Science. 2014; 344:634–638. [PubMed: 24790029]

Wang J, Xu H, Morishima S, Tanabe S, Jishage K, Uchida S, Sasaki S, Okada Y, Shimizu T. Single-channel properties of volume-sensitive Cl− channel in ClC-3-deficient cardiomyocytes. Jpn J Physiol. 2005; 55:379–383. [PubMed: 16441975]

Weiss H, Lang F. Ion channels activated by swelling of Madin Darby Canine Kidney (MDCK) cells. J Membr Biol. 1992; 126:109–114. [PubMed: 1317457]

Wittig I, Beckhaus T, Wumaier Z, Karas M, Schagger H. Mass Estimation of Native Proteins by Blue Native Electrophoresis: Principles and practical hitns. Mol Cell Proteomics. 2010; 9:2149–2161. [PubMed: 20173216]

Zhou HX, Rivas G, Minton AP. Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. Annu Rev Biophys. 2008; 37:375–397. [PubMed: 18573087]

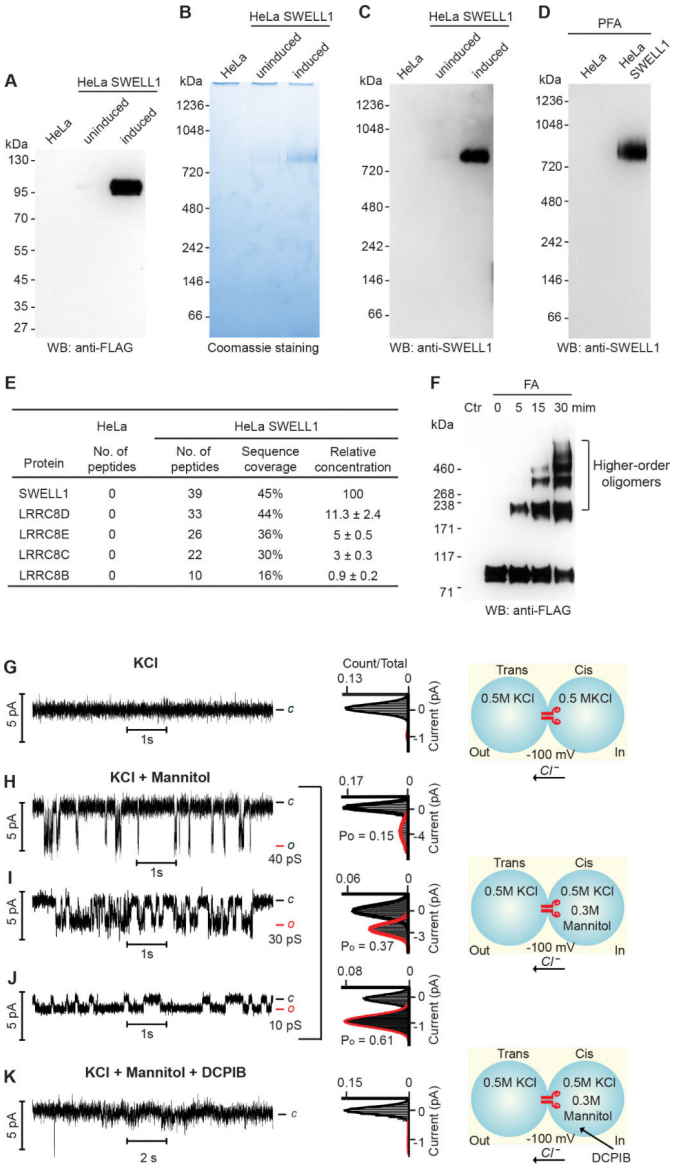


Figure 1. Purification and reconstitution of SWELL1-containing protein complexes

(A) Western blot showing SWELL1-FLAG expression in inducible HeLa cells at low TET concentration (5 ng/ml). **(B–C)** Purified protein samples separated on native gels and visualized by Coomassie staining or western blotting with anti-SWELL1 antibody. **(D)** Western blot of native gel separating proteins purified from paraformaldehyde (PFA)-treated cells. **(E)** A representative mass spectrometry result identifying SWELL1 and its four homologs in the indicated purified protein samples. Relative protein concentrations observed in SWELL1-inducible HeLa cells were expressed as mean ± SEM (n = 3). **(F)** Purified complexes treated with or without formaldehyde (FA) for the indicated time, separated on a denaturing gel and detected with anti-FLAG antibody. Ctr, control. **(G–J)** Single-channel currents of reconstituted complexes in lipid bilayers at −100 mV and all-point current histograms for **(G)** isotonic (n=15) and **(H–J)** hypotonic conditions generated by asymmetric mannitol (n>30) as illustrated (right). **(K)** Blockade of currents after injection of

DCPIB (40 μM final) into *cis* droplet. *c* and *o* indicate closed and open states. Single-channel conductance (γ) in panels **H–J** are indicated below *o*. See Figures S1–S3, Table S1–S2.

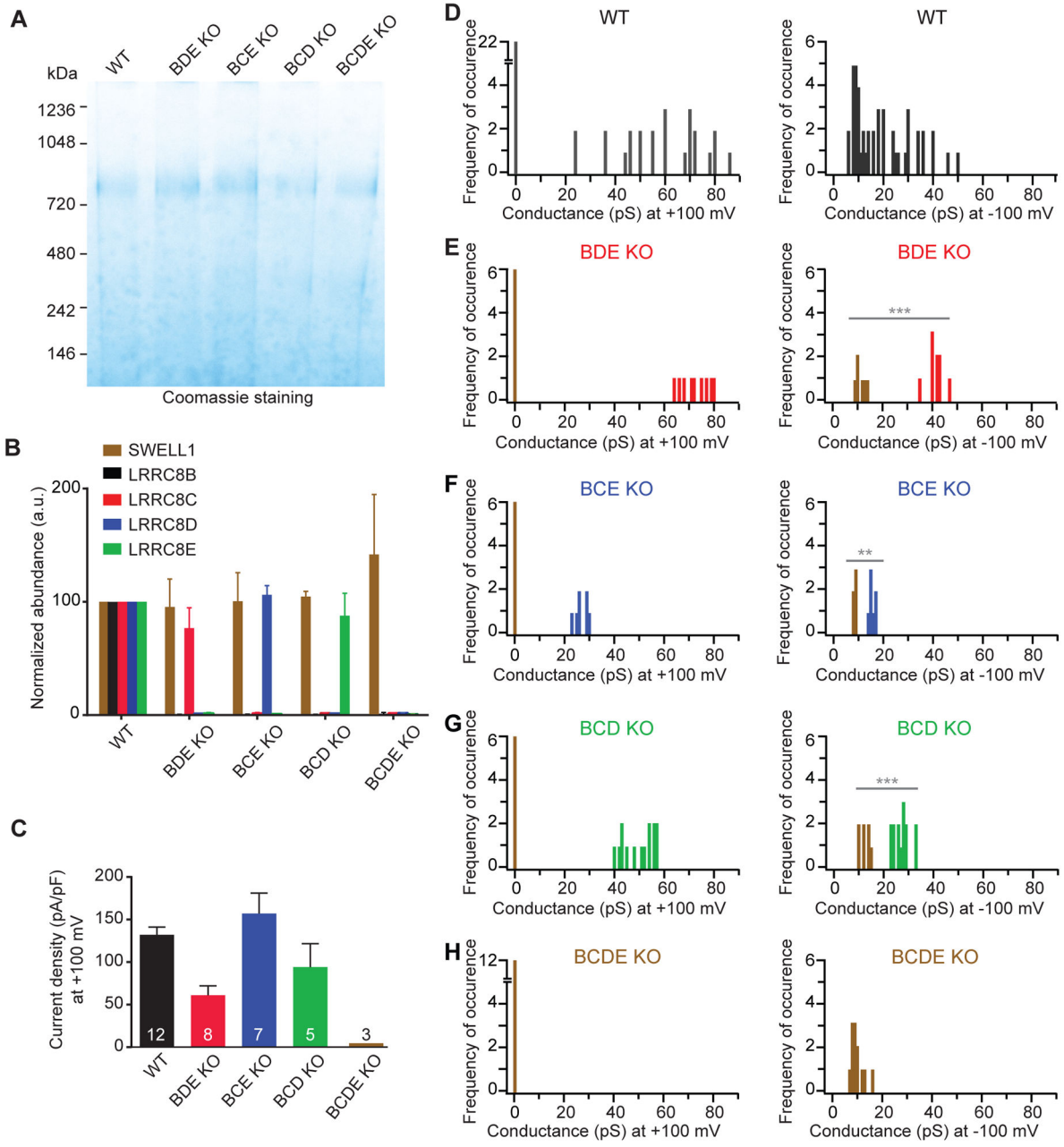


Figure 2. Purified LRRC8 subunit combinations form ion channels with distinct channel conductance in droplet lipid bilayers

(A) Purified protein samples from WT and *LRRC8* triple (BDE, BCE, BCD)- and quadruple (BCDE)-KO SWELL1-inducible HeLa cells separated on a native gel and visualized by Coomassie staining. **(B)** Abundance of each LRRC8 protein normalized to WT (mean ±SEM, n=2) estimated by mass spectrometry. **(C)** Whole-cell current densities of maximally activated $I_{Cl,swell}$ in WT and KO HeLa cells. **(D–H)** Hypotonicity-induced γ of reconstituted complexes after purification from **(D)** WT HeLa, **(E–G)** triple KO HeLa, and **(H)** quadruple KO HeLa cells. At −100 mV, large conductance events were observed in samples from triple-KO cells that differed significantly from the small conductance events apparent in all

samples including those from quadruple-KO cells (brown bars) (** $p<0.01$, *** $p<0.001$; Student's *t*-test). See Figure S4, Table S3.

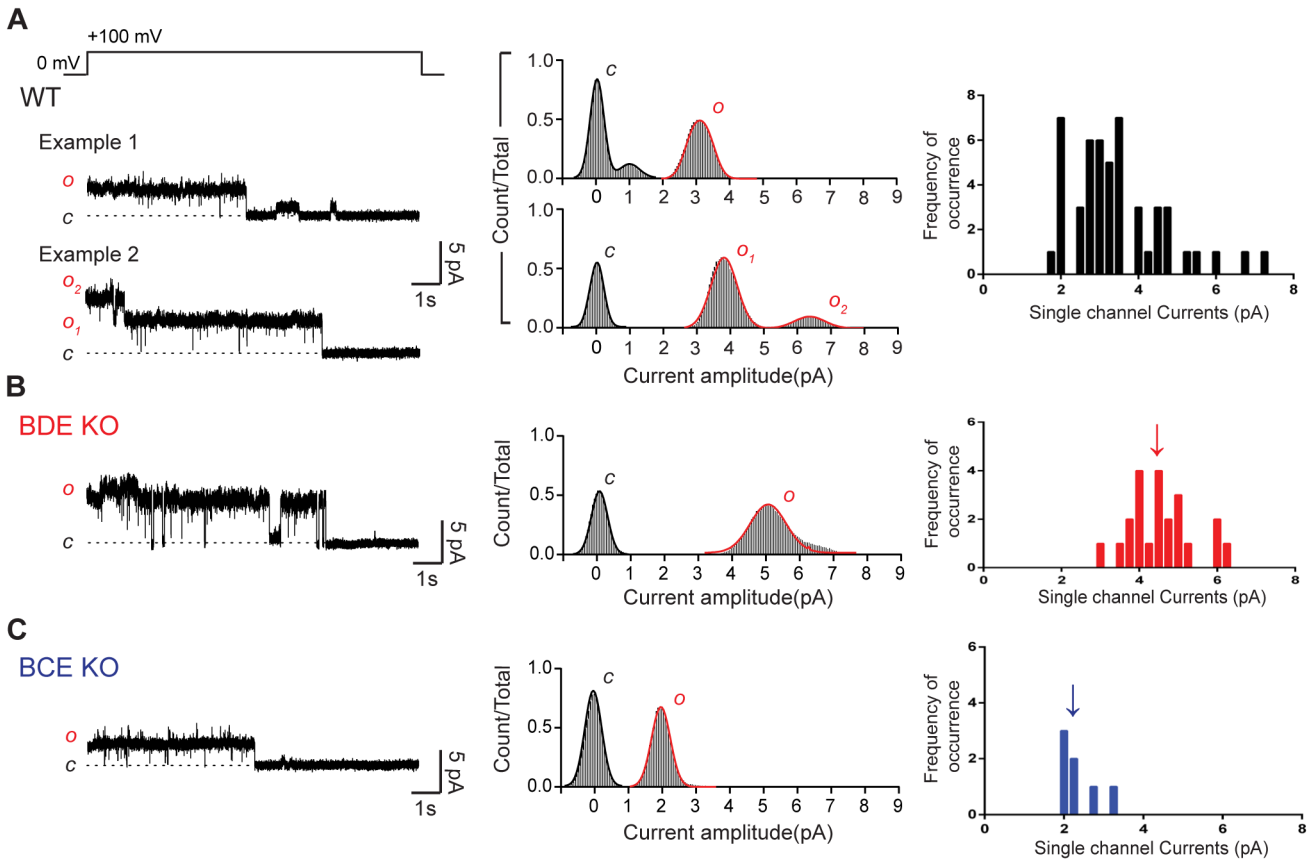


Figure 3. Single-channel currents in cell-attached patches from swollen HeLa cells reveal conductance depends on subunit composition

(A–C) Representative single-channel traces (10 s, left) and all-point current histograms (middle) from WT (**A**), BDE KO (**B**), and BCE KO (**C**) cells recorded near +100mV (pipette potential −100mV). Right: Frequency distribution of current amplitudes observed in single patches from separate cells. (**A**) A wide range of single-channel current amplitudes was observed from WT HeLa cells (n=19, right) (Example 1: 3.0 pA; Example 2: 2.5 pA and 3.8 pA). Background channel activity was present in Example 1 (0.9 pA). Voltage protocol indicated above. **(B)** Current amplitudes of channels composed of SWELL1 and LRRC8C were 4.6±0.2 pA (mean (arrow) ±SEM, n=22 from 11 patches). **(C)** Current amplitudes of channels composed of SWELL1 and LRRC8D were 2.5±0.2 pA (n=7 from 5 patches). See Figure S5, Table S4.

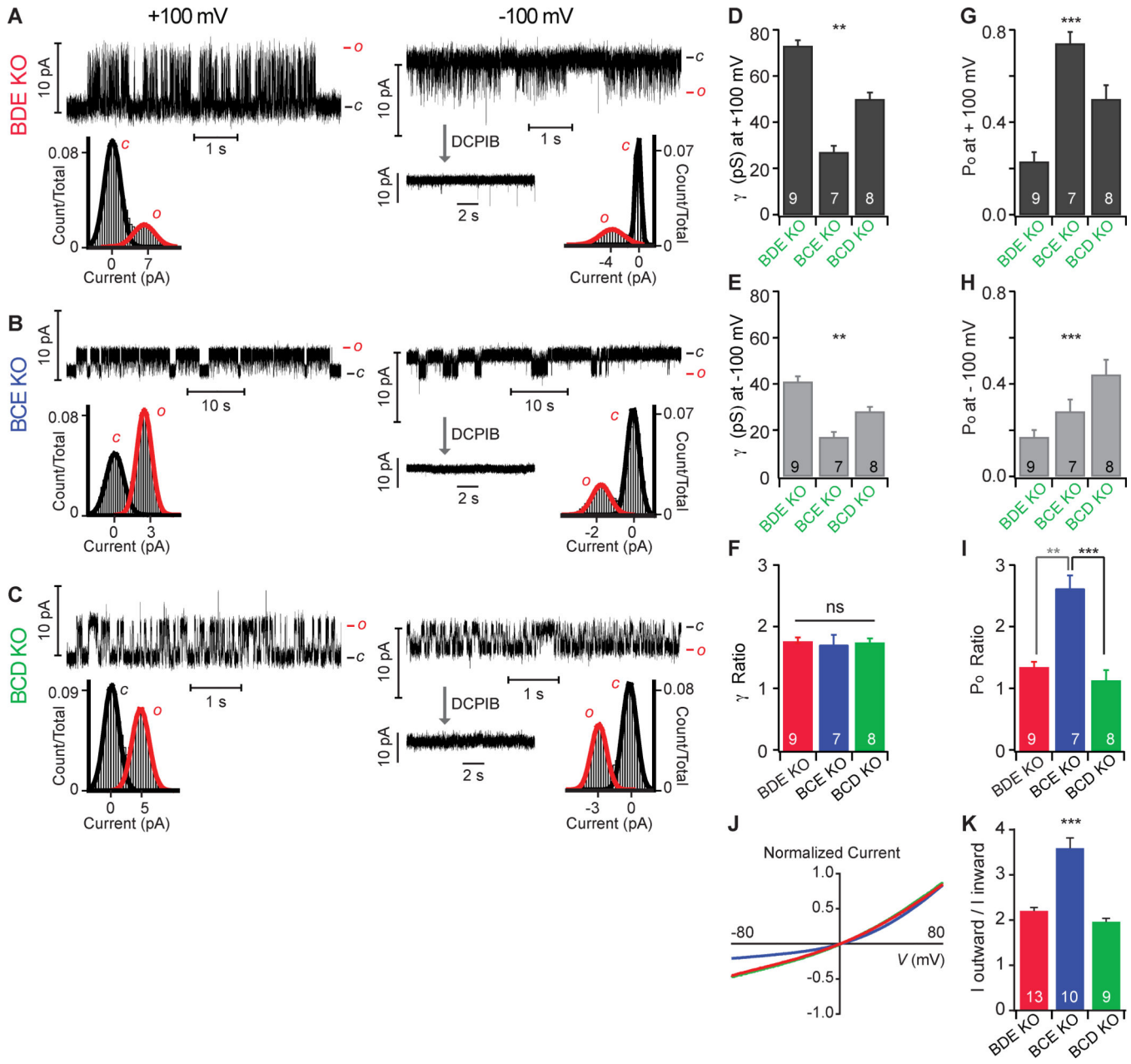
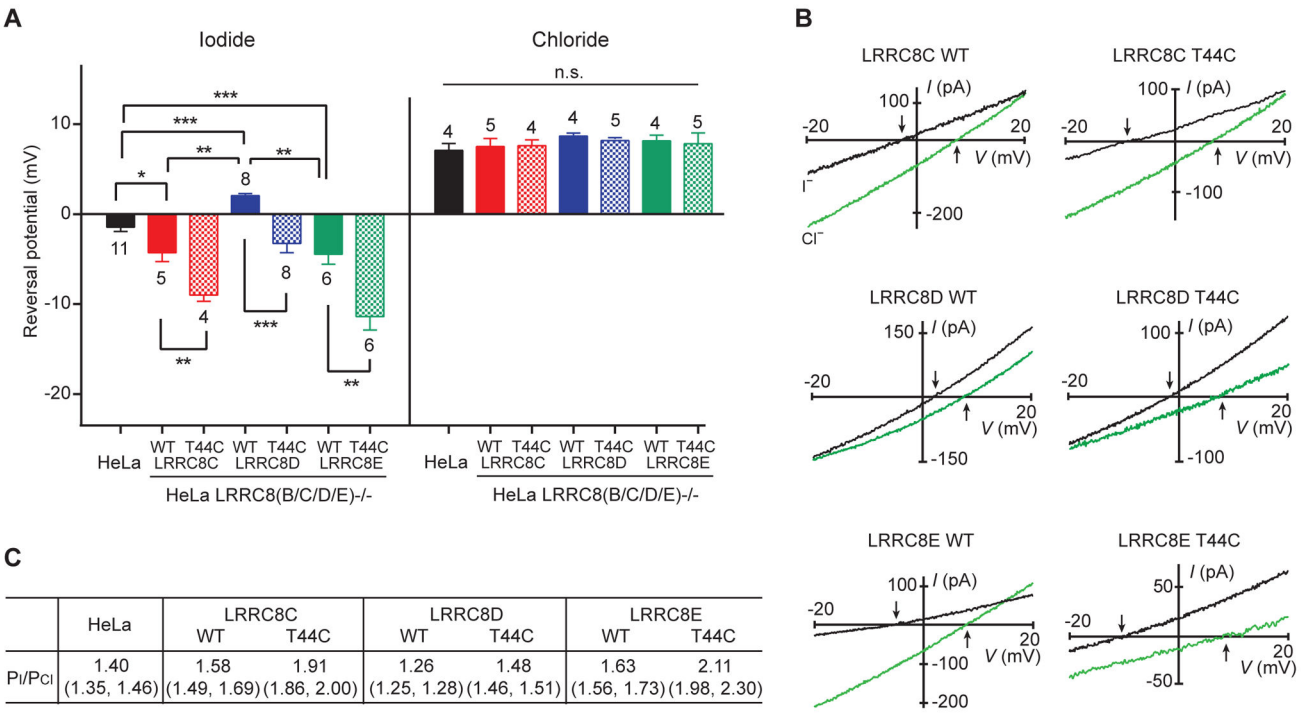


Figure 4. LRRC8 subunit combinations determine VRAC outward rectification

(A–C) Single-channel recordings and all-point current histograms of SWELL1-containing complexes purified from the indicated *LRRC8* KO HeLa cells. Inset: block by DCPIB injection (40 μM final) into the *cis* droplet. **(D–F)** The γ of purified SWELL1-containing complexes and γ ratio (+V:−V)**. (G–I)** P_o and P_o ratio (+V:−V); P<0.01 for A+C vs. A+D and P<0.001 for A+E vs. A+D heteromers. P_o ratio for A+C vs. A+E heteromers was not significantly different. **(J)** Representative leak-subtracted whole-cell hypotonicity-induced currents for *LRRC8(B/D/E)*$^{-/-}$ (red), *LRRC8(B/C/E)*$^{-/-}$ (blue), and *LRRC8(B/C/D)*$^{-/-}$ (green) normalized to the maximum current at +100 mV and centered at V_{rev}. **(K)** Ratio of whole-cell current at ±80mV from V_{rev} for the indicated cells reveals stronger outward

rectification for $I_{Cl,swell}$ in BCE KO compared to other KO cells. Error bars indicate SEM. Significant differences (1- way ANOVA test) are shown: *** ($p<0.001$), ** ($p<0.01$). See Figure S6.



C

	HeLa	LRRC8C WT	LRRC8C T44C	LRRC8D WT	LRRC8D T44C	LRRC8E WT	LRRC8E T44C
P_I/P_{Cl}	1.40 (1.35, 1.46)	1.58 (1.49, 1.69)	1.91 (1.86, 2.00)	1.26 (1.25, 1.28)	1.48 (1.46, 1.51)	1.63 (1.56, 1.73)	2.11 (1.98, 2.30)

Figure 5. T44C mutations in LRRC8 subunits alter anion selectivity

(A) V_{rev} for $I_{Cl,swell}$ recorded from parental or *LRRC8(B/C/D/E)*$^{-/-}$ HeLa cells expressing WT or T44C LRRC8 subunits was determined in the presence of I^- or Cl^- as the only extracellular anion. Bars represent mean±SEM (**P*<0.05; ***P*<0.01; ****P*<0.001; n.s., not significant; unpaired *t*-test). **(B)** Representative leak-subtracted currents recorded in I^- (black) and Cl^- (green). More negative V_{rev} (arrows) in I^- vs. Cl^- solutions indicates selectivity for I^- over Cl^-. **(C)** P_I/P_{Cl} calculations based on data shown in (**A**); values in parentheses indicate the upper and lower compound SEM.

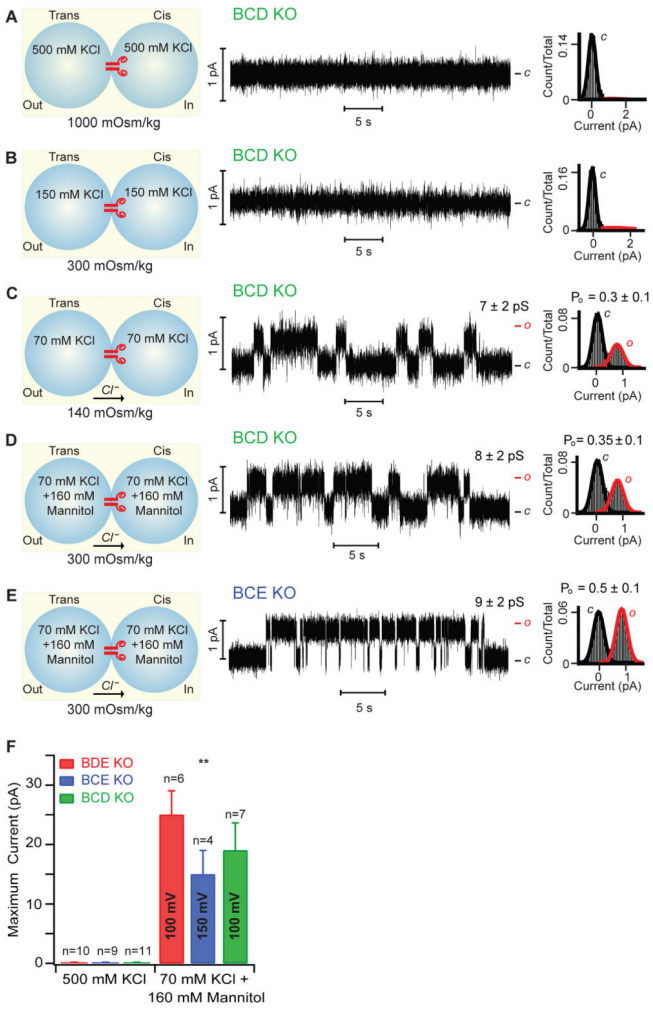


Figure 6. LRRC8 proteins are activated by low ionic strength

(A–C) Single-channel current recordings of the indicated SWELL1-containing complexes after reconstitution in lipid bilayers under different salt conditions (in mM): **(A)** 500 KCl (n=6), **(B)** 150 KCl (n=8), and **(C)** 70 KCl (n=7). **(D–E)** Single-channel currents in 70 mM KCl and mannitol to adjust to physiological osmolality (~300 mOsm/kg) (n=6 and 8 for panels **D** and **E**). **(F)** Maximum currents from multi-channel recordings of indicated complexes in low Γ (n>15). All data were acquired at +100 mV except panel **E** (+150 mV). Significant differences were observed in low Γ for all groups (1-way ANOVA); ** P<0.01. γ and P_o presented in **C**, **D** and **E** are mean±SEM.