

Mechanistic Insights into DCPIB Inhibition of VRAC:

Electrostatic Control and Binding Plasticity

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50 **Abstract**

51 The volume-regulated anion channel (VRAC), formed by LRRC8
52 heteromers, regulates cell volume and is a promising therapeutic target. DCPIB
53 is the most widely used VRAC inhibitor, yet its binding mechanism remains poorly
54 defined. Although structural studies suggested a role of residue R103 in LRRC8A
55 in DCPIB binding, its functional relevance has been unclear. Here, we combined
56 electrophysiology in *Xenopus* oocytes and LRRC8^{-/-} HEK293T cells with
57 molecular dynamics simulations and rational design of DCPIB analogs. Mutations
58 of R103 abolished ATP-dependent block of VRAC but did not affect inhibition by
59 DCPIB or the related inhibitor carbenoxolone (CBX), indicating distinct binding
60 mechanisms. Simulations revealed two DCPIB binding modes: one involving
61 R103 and another engaging K51 and hydrophobic residues. In R103F mutants,
62 DCPIB retained binding via π -stacking and alternative electrostatic contacts,
63 explaining its persistent inhibition. Attempts to disrupt these interactions by
64 mutating K51 led to nonfunctional channels. To further probe the role of
65 electrostatic groups in the binding of DCPIB to VRAC, we synthesized derivatives
66 varying charge and hydrophobicity. Functional assays and steered molecular
67 dynamics (MD) showed that analogs retaining the carboxylate group maintain
68 high-affinity binding, whereas esterified forms exhibit rapid unbinding and
69 reduced potency. Biphenyl substitutions increased binding energy but imposed
70 steric penalties, suggesting a trade-off between affinity and accessibility. Our
71 findings reveal an adaptable binding mechanism for DCPIB, contrasting with
72 described lipid-displacement mechanisms for other VRAC inhibitors, and
73 establish electrostatic complementarity as the primary determinant of high-affinity

74 block. These insights provide a framework for rational design of next-generation
75 VRAC inhibitors with improved specificity.

76

77 **Introduction**

78 Volume-Regulated Anion Channels (VRACs), formed by LRRC8
79 heteromers (Qiu et al., 2014; Stauber, 2015; Voss et al., 2014), mediate chloride
80 and osmolyte flux to maintain cell volume (Jentsch, 2016) and contribute to
81 diverse physiological processes, including apoptosis, immune signaling, insulin
82 secretion, and neurotransmission (Thöne et al., 2025). Dysregulation of VRAC
83 activity has been linked to type 2 diabetes (Kang et al., 2018; Stuhlmann et al.,
84 2018), stroke (Zhou et al., 2020; Yang et al., 2019), cancer (Chen et al., 2019;
85 Jentsch et al., 2016; Planells-Cases et al., 2015), and neuropathic pain (Chu et
86 al., 2023), making these channels attractive therapeutic targets.

87 Despite this potential, VRAC pharmacology remains limited (Figuerola and
88 Denton, 2022; Friard et al., 2017). DCPIB (4-(2-butyl-6,7-dichloro-2-cyclopentyl-
89 indan-1-yl) oxybutyric acid), the most widely used inhibitor, exhibits modest *in*
90 *vivo* potency and poor selectivity, with significant off-target effects (Bowens et al.,
91 2013; Zuccolini et al., 2022). Moreover, its binding mechanism is incompletely
92 understood. Cryo-EM structures have provided snapshots of LRRC8 channels
93 and DCPIB-bound states (Kefauver et al., 2018; Kern et al., 2019; Kern et al.,
94 2023), but the functional relevance of specific interactions proposed from
95 structural data remains to be clarified in physiologically relevant heteromers. A
96 key unresolved issue is whether residues at the extracellular constriction,
97 particularly the conserved arginine ring centered on LRRC8A R103, are required
98 determinants of DCPIB recognition and block in native-like channel assemblies
99 (Kefauver et al., 2018; Liu et al., 2023)

100 Large-pore channels that share ancestry with LRRC8, notably pannexins,
101 provide a useful comparative framework for understanding inhibitor binding
102 (Abasal and Zardoya, 2012). PANX1 and PANX2 possess wide extracellular
103 vestibules but differ markedly in gating and pharmacology: PANX1 is sensitive to
104 carbenoxolone (CBX) (Michalski et al., 2020; Qiu and Dahl, 2009; Hussain et al.,
105 2024), whereas PANX2 is resistant to CBX yet is inhibited by DCPIB (He et al.,
106 2023). This cross-reactivity has been linked to electropositive motifs at the
107 selectivity filter (R103 in LRRC8A; R89 in PANX2) that could attract anionic
108 ligands. Notably, mutagenesis at the PANX2 ring abolishes DCPIB inhibition (He
109 et al., 2023), whereas analogous manipulations in LRRC8A have yielded mixed
110 outcomes (Kefauver et al., 2018), underscoring potential differences in binding
111 plasticity between these channel families.

112 In recent years, several screening approaches have identified unexpected
113 VRAC modulators (Figuroa and Denton, 2022, 2021; Friard et al., 2019, 2017;
114 Jeon and Namkung, 2019; Xu et al., 2023; Sato-Numata et al., 2025), including
115 drugs originally developed for unrelated targets such as pranlukast, zafirlukast
116 (Figuroa et al., 2019), and dicumarol (Chu et al., 2023). These findings, together
117 with structural and functional studies, suggest that these VRAC inhibitors often
118 act through complex mechanisms involving extracellular constrictions or
119 transmembrane fenestrae, and possibly lipid displacement (Yamada et al., 2025,
120 2021; Kern et al., 2019; Gunasekar et al., 2022). However, the relevance of these
121 proposed binding sites in heteromeric channels remains debated.

122 Here, we aim to address this knowledge gap by integrating structural
123 modeling, mutagenesis, and pharmacological profiling to clarify the mechanisms
124 of VRAC inhibition.

125

126 **Materials and methods**

127 *Molecular biology*

128 Plasmid constructs were generated using standard recombinant DNA
129 techniques, including polymerase chain reaction and the Multisite Gateway
130 cloning system (Invitrogen, Carlsbad, CA). All constructs were verified by Sanger
131 sequencing to confirm sequence integrity.

132 For expression in *Xenopus laevis* oocytes, plasmids encoding human
133 LRRC8 isoforms were linearized with NotI and transcribed into complementary
134 RNA (cRNA) using the mMessage mMachine SP6 transcription kit (Ambion,
135 Waltham, MA). We synthesized cRNA for wild-type (WT) and mutant variants of
136 LRRC8A tagged with Venus fluorescent protein (VFP), as well as LRRC8E
137 tagged with monomeric Cherry (mCh).

138 For expression in LRRC8-deficient HEK293T cells, co-expression of
139 LRRC8A and LRRC8E was achieved using bicistronic pcDNA3.1 constructs.
140 These constructs contained both coding sequences separated by a T2A
141 ribosome-skipping peptide to ensure equimolar expression of the untagged
142 isoforms (Liu et al., 2017).

143

144 *Organic Synthesis*

145 All chemicals were purchased from commercial sources and were used as
146 received unless otherwise stated. All reactions were performed under argon in
147 dried glassware, unless otherwise stated. Microwave-irradiated reactions were
148 carried out using a Biotage Initiator Classic. Column chromatographies were

149 carried out on commercial silica gel. Flash column chromatographies were
150 carried out using an Isolera Prime Biotage equipped with dual UV detection. The
151 ¹H NMR spectra were recorded on 400 MHz or 600 MHz NMR spectrometers.
152 The ¹³C NMR spectra were recorded at 100 MHz or 150 MHz. The High-
153 Resolution Mass Spectrometry was performed by the Mass Spectrometry and
154 Molecular Characterization Unit of the Scientific and Technological Centers at the
155 Universitat de Barcelona (CCiTUB). A more detailed description could be found
156 in the Supporting Information section.

157

158 *Oocyte preparation and injection*

159 Stage V–VI oocytes were surgically harvested from *Xenopus laevis* frogs
160 (Xenopus Express, Vernassal, France). Prior to surgery, animals were
161 anesthetized with tricaine (ethyl 3-aminobenzoate methane sulfonate; Sigma-
162 Aldrich, St. Louis, MO) at a concentration of 1 g/L. Post-operative recovery and
163 care were provided in accordance with institutional and European guidelines. All
164 procedures complied with the European Community Directive 2010/63/EU on the
165 protection of animals used for scientific purposes and were approved by the
166 Ethics Committee for Animal Experimentation of the University of Barcelona and
167 the Government of Catalonia (protocol number 371/17).

168 Oocytes were enzymatically defolliculated by incubation in ORII solution
169 containing (in mM): 90 NaCl, 2 KCl, 1 MgCl₂, and 10 HEPES (pH 7.4, adjusted
170 with Tris-HCl), supplemented with 2 mg/mL collagenase D (Roche) under gentle
171 agitation for 90 minutes at 17°C. Following defolliculation, oocytes were
172 maintained at 17°C in Barth's solution containing (in mM): 88 NaCl, 1 KCl, 0.82

173 MgSO₄, 0.41 CaCl₂, 0.33 Ca(NO₃)₂, 2.4 NaHCO₃, and 10 HEPES (pH 7.4,
174 adjusted with Tris), supplemented with 10 mg/L gentamycin.

175 Each oocyte was injected with 50 nL of cRNA solution using the
176 Inject+Matic microinjector (Geneva, Switzerland), delivering 7 ng of each LRRC8
177 subunit. Electrophysiological recordings were performed in ND96 solution
178 containing (in mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES (pH 7.4,
179 adjusted with Tris).

180 The voltage-clamp protocol consisted of an initial depolarizing pulse of +90
181 mV for 100 ms, followed by 1500 ms voltage steps ranging from +90 mV to -150
182 mV in 20 mV increments, and a final tail pulse of -90 mV for 100 ms.

183

184 *Cell culture*

185 HEK293 LRRC8^{-/-} cells (HEK293-5X-KO), lacking all five LRRC8 subunits
186 (Lutter et al., 2017), were generously provided by Dr. Thomas Jentsch. Cells were
187 maintained in Dulbecco's Modified Eagle Medium (DMEM; Pan Biotech,
188 Aidenbach, Germany) supplemented with 10% fetal bovine serum, 1% penicillin-
189 streptomycin, and 1% L-glutamine. Cultures were incubated at 37°C in a
190 humidified atmosphere containing 5% CO₂. Cells were grown on standard plastic
191 tissue culture dishes and passaged every 3–4 days. Transfections were
192 performed as previously described (Ferrera et al., 2021), using varying amounts
193 of cDNA depending on the experimental conditions. Whole-cell recordings were
194 conducted 24–36 hours post-transfection.

195

196 *Patch-clamp recording*

197 Whole-cell voltage-clamp recordings were performed as described
198 (Ferrera et al., 2021). Series resistance (R_s) ranged from 2 to 8 M Ω (mean $\pm$ SD:
199 5.1 ± 2.8 M Ω) and was not compensated. Cell capacitance (C_m) ranged from 10
200 to 40 pF (mean $\pm$ SD: 22.8 ± 5.3 pF). Cells were held at 0 mV, and stimulation
201 protocols consisted of 50 ms voltage steps to 0, -75, -25, +25, and +75 mV every
202 5 seconds. These steps also served to confirm the absence of significant leak
203 currents, as the expected reversal potential under the ionic conditions used was
204 -25 mV, and VRAC currents displayed slight outward rectification.

205 Experiments involving extracellular application of inhibitors were initiated
206 only after baseline current stabilization (typically monitored for 2 minutes). The
207 standard isotonic extracellular solution contained (in mM): 145 NaCl, 6 KCl, 1.5
208 CaCl_2 , 1 MgCl_2 , 10 HEPES, and 10 glucose (pH 7.4, 310 mOsm). The hypotonic
209 solution contained (in mM): 105 NaCl, 6 CsCl, 1.5 CaCl_2 , 1 MgCl_2 , 10 HEPES,
210 and 10 glucose (pH 7.4, 230 mOsm).

211 The pipette solution used to monitor VRAC activation under hypotonic
212 conditions contained (in mM): 100 potassium gluconate, 40 CsCl, 2 MgCl_2 , 1.9
213 CaCl_2 , 5 EGTA-NMDG, 1 Na_2ATP , and 10 HEPES-NMDG (pH 7.3, 290 mOsm).
214 Free Ca^{2+} concentration was calculated to be ~80 nM. Inhibitors were dissolved
215 directly in the hypotonic extracellular solution.

216 Data were analyzed using Ana software
217 (<http://users.ge.ibf.cnr.it/pusch/programs-mik.htm>) and Prism (GraphPad
218 Software, Inc.). Current amplitudes were quantified as the mean current during
219 the +75 mV step, measured ~10 ms after the voltage change to exclude

220 capacitive artifacts. Data are presented as mean $\pm$ SD from independent HEK
221 cell recordings.

222

223 *Two-Electrode Voltage-clamp recording*

224 Two to three days post-injection, oocytes were subjected to voltage-clamp
225 recordings using either CellWorks (NPI Electronic, Tamm, Germany) or the
226 custom acquisition software GePulse ([http://users.ge.ibf.cnr.it/pusch/programs-](http://users.ge.ibf.cnr.it/pusch/programs-mik.htm)
227 [mik.htm](http://users.ge.ibf.cnr.it/pusch/programs-mik.htm)), in combination with Turbo TEC-03X or TEC-05X amplifiers (NPI
228 Electronic). Recordings were performed in isotonic ND96 solution containing (in
229 mM): 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES (pH 7.4, adjusted with
230 Tris).

231 For standard current traces, the voltage protocol included a 100 ms pulse
232 to -70 mV, followed by 3000 ms steps from -90 mV to +70 mV in 20 mV
233 increments, and a final tail pulse of 300 ms at -70 mV. In experiments testing
234 inhibitors, recordings were initiated only after stable VRAC/LRRC8-mediated
235 currents were established in isotonic medium.

236 For single-concentration inhibitor assays, the protocol consisted of 200 ms
237 pulses to +60 mV every 10 seconds, with recordings comprising 1 minute in
238 isotonic medium followed by 5 minutes in the inhibitor-containing isotonic
239 solution. For dose-response experiments, the same voltage protocol was used,
240 with 2 minutes in isotonic medium followed by sequential 3-minute exposures to
241 increasing inhibitor concentrations.

242 Inhibitory effects were quantified as described (Gaitán-Peñas et al., 2016).
 243 Briefly, the steady-state current ($I_{\text{inhibitor}}$) at each concentration was estimated by
 244 fitting an exponential decay to the time course of current inhibition and
 245 extrapolating to infinite time. The normalized current ($I_{\text{inhibitor}}/I_0$) was fitted to the
 246 Hill equation:

$$252 \quad p_u = p_\infty + \frac{1 - p_\infty}{1 + \left(\frac{[Inhib]}{IC_{50}}\right)^n}$$

247 where p_u is the unblocked current fraction, $[Inhib]$ is the inhibitor concentration,
 248 IC_{50} is the half-maximal inhibitory concentration, n is the Hill coefficient, and p_∞
 249 accounts for residual leak currents. Nonlinear regression fitting was performed in
 250 Prism using the four-parameter variable slope model, with bottom and top
 251 constraints set to 0 and 1, respectively.

253

254 *Structural Modeling of VRAC channels*

255 The VRAC channel was modeled based on the cryo-EM structure reported
 256 by Rutz et al. (PDB ID: 8B42; resolution: 6.60 Å), which comprises a heteromeric
 257 hexamer formed by four LRRC8A and two LRRC8C subunits from *Mus musculus*
 258 (Rutz et al., 2023). Homology models of human LRRC8C and LRRC8E, including
 259 their N-terminal domains, were generated using the Robetta server (Baek et al.,
 260 2021), based on the high-resolution human LRRC8A structure (PDB ID: 7XZH;
 261 2.78 Å) (Liu et al., 2023) using only the transmembrane helices (position used for
 262 alignment: 15-58, 92-174, 236-404). The N-terminal domain (NTD) was poorly
 263 predicted by the Robetta webserver and was modelled using the human LRRC8A

264 structure (PDB id 7XZH) as a reference and manually mutating the sequence to
265 the LRRC8C and LRRC8E. The resulting models were selected according to the
266 lowest RMSD values of the C α atoms of the transmembrane helices and any
267 diverging loop was manually refined. The resulting monomers were energy-
268 minimized prior to integration into the hexameric complex. Only the
269 transmembrane domains were retained, excluding the cytosolic leucine-rich
270 repeat regions (**Figure S1**). The binding mode of DCPIB was inferred by
271 superimposing the LRRC8A–DCPIB complex structure (PDB ID: 6NZW; 3.21 Å)
272 (Kern et al., 2019) (**Figure S2**). Binding poses of the novel inhibitors were
273 modeled by aligning their indanone core to that of DCPIB. Protein atoms were
274 described using the Amber14SB force field (Maier et al., 2015). Ligands were
275 parameterized using the GAFF force field with RESP charges (HF/6-31G(d)) via
276 the Antechamber module of AmberTools (Case et al., 2023). Lipids were modeled
277 using the Amber Lipid21 force field (Dickson et al., 2022), and counterions were
278 described using Joung and Cheatham parameters (Joung and Cheatham, 2009).

279 Hexameric LRRC8A:LRRC8E (4:2), LRRC8A:LRRC8C (4:2) and LRRC8A
280 complexes were embedded into a pre-equilibrated 125 × 125 Å² lipid bilayer using
281 Charmm-GUI (Jo et al., 2008; Lee et al., 2016, 2020). The membrane
282 composition mimicked that of human cell membranes: the outer leaflet contained
283 43% cholesterol (CHL), 49% POPC, and 8% POPE; the inner leaflet contained
284 35% CHL, 21% POPC, 31% POPE, and 13% POPS (**Table S1**). A 60 Å water
285 layer was added on both sides, and K⁺/Cl⁻ ions were included to reach 0.15 M
286 ionic strength. The final system dimensions were 125 × 125 × 160 Å³, comprising
287 ~232,800 atoms.

288

289 *Molecular Dynamics Simulations*

290 Each system underwent energy minimization using 3,500 steps of
291 steepest descent followed by 4,500 steps of conjugate gradient. Thermalization
292 was performed in six 1 ns stages, gradually increasing the temperature from 50
293 K to 303.5 K. Lipid restraints were reduced from 5.0 to 0.1 kcal·mol⁻¹·Å⁻², while
294 protein and ligand atoms were restrained with a force constant of 10
295 kcal·mol⁻¹·Å⁻². Equilibration followed with 6 ns of restrained protein-ligand
296 complex (2.5 kcal·mol⁻¹·Å⁻²) and 10 ns with ligand-only restraints (1.0
297 kcal·mol⁻¹·Å⁻²), totaling 22 ns.

298 Production simulations consisted of five independent 500 ns trajectories
299 per system, yielding a total of 50 μs of simulation time. Simulations employed a
300 4 fs timestep using hydrogen mass repartitioning, SHAKE constraints on
301 hydrogen bonds, periodic boundary conditions (Case et al., 2023), Langevin
302 thermostat (303.5 K, 1 ps⁻¹ collision frequency), anisotropic pressure coupling,
303 and particle mesh Ewald for long-range electrostatics with a 9 Å cutoff (**Figures**
304 **S3 to S27**).

305

306 *Steered Molecular Dynamics Simulations*

307 To estimate the energetic cost of ligand unbinding from the selectivity filter,
308 reduced models were constructed containing all residues within 30 Å of the
309 DCPIB binding site. These truncated systems (~47,800 atoms) were capped and

310 minimized using a similar process to the Dynamic Undocking protocol (DUck)
311 (Ruiz-Carmona et al., 2017). Thermalization was performed in four 1 ns stages
312 (100 → 303 K) under NVT conditions, followed by 4 ns of NPT equilibration using
313 the Berendsen barostat and Langevin thermostat. C α atoms were restrained
314 throughout equilibration (5.0 kcal·mol⁻¹·Å⁻²) to preserve structural integrity in the
315 absence of a membrane.

316 Ligand unbinding was simulated using a distance-based collective variable
317 (CV), defined as the Euclidean distance between the center of mass of the
318 indanone scaffold and the backbone atoms of residues T48 (LRRC8A), M48
319 (LRRC8C), and T48 (LRRC8E) (**Figure S30**). The CV was increased from ~18–
320 19 Å (bound state) to 33 Å (unbound state) using a pulling rate of 0.5 Å·ns⁻¹ and
321 a force constant of 500 kcal·mol⁻¹·Å⁻², ensuring compliance with the stiff spring
322 approximation. A hundred replicates were performed for each protein-ligand
323 system in LRRC8A:LRRC8E heteromer.

324

325 *Trajectory Analysis*

326 Standard trajectory analysis were performed using CPPTRAJ (Roe and
327 Cheatham, 2013), including monitoring root mean square deviation (RMSD),
328 selected interatomic distances, ion density and PMF profiles. Density grids were
329 calculated with CPPTRAJ using evenly spaced 100 frames extracted from the
330 last 100 ns of simulation time of each replicate, accounting for 500 frames for
331 each simulated system.

332

333 *Statistics*

334 All quantitative data are presented as mean $\pm$ SD. Statistical significance
335 was determined using GraphPad Prism. For comparison between two groups, a
336 two-tailed unpaired Student's t-test was used. For multiple group comparisons,
337 one-way ANOVA followed by Tukey's post hoc test was applied. The statistical
338 test used in each case is indicated in the respective figure legend.

339

340 *Online supplemental materials*

341 Two supplementary files are added: molecular modelling and chemistry.
342 The supplementary information of molecular modelling contains 30
343 supplementary figures and 3 supplementary tables. **Figure S1** contains the
344 overview of the computational models generated in this work. **Figure S2** shows
345 the comparison of the density of the DCPIB between CryoEM and Molecular
346 Dynamics simulations. **Figures S3 to S29** includes trajectory analysis of the
347 unbiased molecular dynamics simulations. **Figure S30** contain information used
348 for the steered MD simulations. **Table S1** contains the membrane composition of
349 the transmembrane model. **Table S2** includes a detailed summary of all the
350 molecular dynamics simulations. **Table S3** details the population of each binding
351 mode in the molecular dynamics simulation of all the ligand-protein systems.

352 Finally, the supplementary information of chemistry contains the complete
353 synthetic procedures and structural characterization for the DCPIB derivatives
354 (Schemes A and B of the synthetic preparation).

355 **Results**

356

357 *R103F mutation abolishes ATP block but preserves DCPIB inhibition in LRRC8-*
358 *^{-/-} HEK293T cells*

359 Cryo-EM structures of LRRC8/VRAC channels revealed that a ring of
360 arginines (R103) at the N-terminal tip of the pore helix forms the narrowest
361 extracellular constriction (Kefauver et al., 2018). This electropositive ring has
362 been proposed to influence permeation and gating by shaping the electrostatic
363 environment, potentially affecting interactions with charged modulators such as
364 ATP. To test this hypothesis, we examined the effect of substituting R103 in
365 LRRC8A by phenylalanine on the pharmacological profile of VRAC.

366 Whole-cell patch-clamp recordings were performed in *LRRC8^{-/-}* HEK293T
367 cells transiently transfected with bicistronic constructs encoding LRRC8A (WT or
368 R103F) and LRRC8E separated by a T2A sequence to ensure co-expression.
369 Under hypotonic conditions, both WT and R103F-containing heteromers
370 generated robust VRAC currents, confirming that the mutation does not impair
371 channel activation. Application of 2 mM ATP markedly inhibited outward currents
372 in WT channels, whereas this effect was strongly attenuated in R103F-containing
373 channels (**Figure 1A**). In contrast, inhibition by the VRAC blocker DCPIB (10 μ M)
374 was comparable between WT and R103F channels, with no significant
375 differences detected (**Figure 1B**). Thus, R103F selectively disrupts ATP-
376 mediated inhibition while preserving DCPIB sensitivity.

377 These findings are consistent with previous observations in LRRC8A +
378 LRRC8C assemblies (Kefauver et al., 2018), indicating that the contribution of

379 R103 to ATP inhibition is preserved across heteromeric contexts, whereas DCPIB
380 inhibition remains unaffected by this mutation.

381

382 *Testing DCPIB inhibition in Xenopus oocytes expressing LRRC8A and LRRC8E*
383 *mutants*

384 To gain further insight into the mechanism of DCPIB inhibition, we
385 employed our *Xenopus* oocyte expression system, which provides constitutively
386 active VRAC currents under isotonic conditions and is well suited for
387 pharmacological screening (Gaitán-Peñas et al., 2016, 2018). This approach
388 allowed us to test whether mutations at the N-terminal tip of the pore helix,
389 previously implicated in ATP block, could also influence sensitivity to DCPIB or
390 other canonical VRAC inhibitors such as carbenoxolone (CBX). We introduced
391 two substitutions at R103 in LRRC8A, phenylalanine (R103F) and glutamine
392 (R103Q), to probe the nature of potential interactions with DCPIB.

393 We first co-expressed LRRC8A-VFP (WT, R103F, or R103Q) with
394 LRRC8E-mCh and confirmed robust currents in all combinations (**Figure 2A**).
395 WT channels exhibited typical outward current inactivation at positive potentials,
396 whereas this feature was abolished in R103F- and R103Q-containing
397 heteromers, consistent with the proximity of R103 to the KYD motif involved in
398 gating (Ullrich et al., 2016). When we assessed DCPIB inhibition, single-point
399 measurements at 20 μ M revealed similar inhibition for WT, R103F, and R103Q
400 ($\approx$ 60–70%; ns; **Figure 2B**), indicating that neither mutation significantly alters
401 DCPIB sensitivity. In contrast, ATP-mediated block was strongly reduced in
402 R103F channels compared to WT, confirming the critical role of R103 in ATP

403 sensitivity and gating (**Figure 2C**). Finally, CBX sensitivity was analyzed for
404 R103F, and concentration-response analysis revealed no significant difference
405 compared to WT ($IC_{50} \approx 12 \mu M$ for WT vs $\approx 11 \mu M$ for R103F; Hill coefficient ≈ 1.5 ;
406 $n = 4$; **Figure 2D**).

407 We then examined homologous residues in LRRC8E (L96 and Q97) and
408 introduced mutations L96A and Q97E, since similar mutations in pannexin 1
409 (W74A and R75E), affect CBX binding (Ruan et al., 2020). Both mutations were
410 functional (**Figure 2E**) and did not affect, or only slightly reduced, DCPIB
411 inhibition at a single concentration (**Figure 2F**). Dose-response analysis for Q97E
412 confirmed no significant change in IC_{50} (WT = $8.59 \mu M$ vs Q97E = $10.95 \mu M$; ns;
413 **Figure 2G**). Collectively, these results indicate that neither R103 in LRRC8A nor
414 residues L96 and Q97 in LRRC8E are critical for DCPIB sensitivity. By contrast,
415 R103 plays a major role in ATP-mediated block and gating, while the contribution
416 of L96 and Q97 to CBX sensitivity was not assessed.

417

418

419 *Molecular dynamics simulations uncover dual DCPIB binding modes and explain*
420 *persistence of inhibition in R103F mutants*

421 Since electrophysiological experiments indicated that mutations at the
422 pore helix in LRRC8A or LRRC8E did not alter DCPIB sensitivity, we
423 hypothesized that DCPIB may interact with VRAC through a pattern of
424 interactions distinct from that of ATP. To explore this possibility, we performed
425 extensive molecular dynamics simulations using the cryo-EM structure of

426 LRRC8A/LRRC8C heteromers (PDB: 8B41; (Rutz et al., 2023)) and homology
427 models of LRRC8E generated with Robetta (Baek et al., 2021) (**Figure 3A and**
428 **Figure S1**). Channels were assembled with a 4:2 stoichiometry
429 (LRRC8A:LRRC8C or LRRC8A:LRRC8E) (Rutz et al., 2023), and ligand binding
430 was examined in both the WT (**Figure S2**) and R103F complexes. Each condition
431 was simulated in five independent 500-ns trajectories, totaling 15 μ s of sampling
432 (**Figures S3 to S10**).

433 Simulations confirmed that ATP remains stably anchored at the selectivity
434 filter of WT channels throughout all replicates (**Figures S4 to S5**). Its phosphate
435 groups form persistent salt bridges with R103 sidechains, while the ribose and
436 adenine moieties interact with D104 and the backbone of L105 (**Figure 3B**). In
437 contrast, the R103F mutation profoundly altered the electrostatic landscape of
438 the vestibule, eliminating positive charges and allowing cations to penetrate the
439 pore region. The destabilization of ATP binding led to ATP dissociation in all
440 replicates (**Figure S9**), providing a mechanistic explanation for the loss of ATP-
441 mediated inhibition observed experimentally (**Figures 1A and 2A**).

442 DCPIB behaved differently. In WT channels, the inhibitor adopted two
443 distinct poses: one maintaining salt bridges with R103 and another shifted inward
444 to interact with K51, while its hydrophobic indanone moiety engaged residues
445 such as L105 (LRRC8C) or L96 (LRRC8E) (**Figure 3C and 3D and Figures S6**
446 **to S7**). Both configurations effectively occluded the pore, and their calculated
447 densities matched experimental cryo-EM maps (PDB IDs 6NZW and 6NZZ; (Kern
448 et al., 2019)) (**Figure S2**). Remarkably, in R103F mutants, DCPIB retained stable
449 binding by exploiting alternative interactions, forming π -stacking with F103 and

450 maintaining contacts with K51 or nearby cations (**Figure 3E** and **Figures S10**).
451 This adaptability explains why DCPIB inhibition persists despite the loss of R103,
452 in sharp contrast to ATP, which lacks compensatory interactions due to its highly
453 polar scaffold.

454 Interestingly, DCPIB also adopts these binding modes in the homomeric
455 LRRC8A assemblies. In the wild-type LRRC8A complex, DCPIB establishes a salt-
456 bridge and a cation- π interaction with R103 sidechains as it is not able to engage
457 any hydrophobic residues. In the mutant R103F LRRC8A complex, DCPIB does
458 not establish stable interactions with the K51 due to the bulk of the F103
459 sidechains, thus leading to a weakened binding mode (**Figure S26** and **S27**), in
460 agreement with experimental data (Yamada et al., 2021).

461

462 *Structural and functional analysis of selectivity filter mutations reveals DCPIB*
463 *binding plasticity*

464 To further investigate the structural determinants of DCPIB binding, we
465 first analyzed the electrostatic profile of the selectivity filter of the WT channel
466 using APBS calculations (Jurrus et al., 2018; Baker et al., 2001) (**Figure 4A, 4B**).
467 The permeation pathway displays a strongly electropositive electrostatic potential
468 along its axis, consistent with the established preference of LRRC8A:C and
469 LRRC8A:E heteromeric channels for anionic substrates.

470 Based on sequence conservation within the selectivity filter and on the
471 electrostatic features of the binding pocket, we prioritized several substitutions
472 expected to perturb local charge distribution and steric environment: R103F

473 (previously characterized), single K51 substitutions (K51Q), the double mutant
474 K51F-R103F, and T48D, which introduces a negative charge immediately below
475 the filter (**Figure 4A**). These variants were assessed using molecular dynamics
476 simulations and electrophysiological recordings to evaluate their impact on
477 DCPIB sensitivity.

478 Equilibrium MD simulations of WT and mutant LRRC8A:LRRC8C
479 channels (WT, R103F, K51F-R103F, K51Q and T48D), performed in the absence
480 and presence of DCPIB and totaling 10 μ s of sampling, revealed mutation-
481 dependent alterations in ion occupancy profiles (**Figure 4C**). In the apo state, WT
482 channels showed pronounced chloride density between the T48 position and the
483 M1 helix, as well as an additional chloride density near R103, corresponding to
484 the canonical ligand-binding region. This latter density was absent in R103F-
485 containing channels, which instead exhibited potassium density in the inner
486 vestibule, consistent with previously reported reductions in chloride selectivity
487 and shifts in reversal potential (Kefauver et al., 2018).

488 The K51F-R103F double mutant was designed to eliminate the alternative
489 DCPIB binding configuration observed in R103F channels, in which
490 compensatory interactions with K51 partially stabilize ligand binding. APBS
491 calculations and equilibrium MD simulations indicated that this double
492 substitution markedly attenuates the electropositive character of the inner
493 vestibule and alters equilibrium ion distributions (**Figure 4B, 4C**), consistent with
494 a less favorable electrostatic environment for stable anion occupancy. This
495 mutant did not yield measurable currents in electrophysiological recordings and
496 therefore could not be functionally validated for DCPIB sensitivity, precluding

497 direct experimental validation of the predicted binding modes (**Figures S11 and**
498 **S12, Figure 4E**).

499 To isolate the contribution of K51 independently of R103, we next
500 examined single K51 substitutions. Functional recordings of K51Q mutants
501 introduced selectively in LRRC8A or LRRC8E subunits yielded robust currents
502 and retained strong sensitivity to DCPIB (**Figure 4D**). Consistently, equilibrium
503 MD simulations of K51Q-containing channels revealed preservation of the
504 dominant R103-mediated DCPIB binding mode, while alternative configurations
505 remained accessible due to heteromeric contributions (**Figures S15-S16**). These
506 results indicate that individual perturbation of K51 is insufficient to measurably
507 alter DCPIB inhibition under the conditions tested.

508 Introduction of the T48D mutation significantly modified the local
509 electrostatic landscape below the selectivity filter (**Figure 4B**) and reduced stable
510 chloride and potassium occupancy in the vestibule under equilibrium simulation
511 conditions (**Figure 4C**). Functionally, this mutation was associated with a marked
512 loss of outward inactivation, while still supporting measurable macroscopic
513 currents (**Figure 4G**). These observations are consistent with a model in which
514 stable anion occupancy of the selectivity filter, as captured in equilibrium MD
515 simulations, contributes to the stabilization of non-conductive or rectifying states
516 underlying outward inactivation. Destabilization of these ion-occupied
517 configurations by the T48D mutation would impair inactivation, while preserving
518 transient conductive states sufficient to sustain ion permeation under
519 physiological driving forces.

520

521 When DCPIB was placed in the filter *in silico*, it remained stably bound in
522 most replicates of the T48D through interactions with R103 and K51 sidechains
523 that remain intact in this construct (**Figure 4F**; **Figures S13-S14**).
524 Electrophysiology recordings corroborated these findings, demonstrating potent
525 DCPIB inhibition of T48D-containing channels (**Figure 4G**), indicating that
526 electrostatic perturbation at position 48 alone is insufficient to confer resistance.

527 Together, these results show that targeted perturbations of the selectivity
528 filter electrostatic environment modulate equilibrium ion occupancy and gating
529 behavior but have limited impact on DCPIB sensitivity. DCPIB binding is
530 preserved through alternative and compensatory interactions networks within the
531 filter, underscoring a high degree of binding plasticity and explaining the
532 resilience of pharmacological inhibition across diverse mutational backgrounds.

533

534 *Chemical analogs confirm electrostatic interactions as key determinants of VRAC*
535 *block*

536 Given the limited ability of selectivity filter mutations to disrupt DCPIB
537 inhibition, we therefore next explored whether systematic modifications of the
538 ligand itself could more effectively dissect the electrostatic and steric interactions
539 governing VRAC block.

540 To explore the role of chemical motifs in DCPIB inhibition of LRRC8
541 channels, we designed five DCPIB derivatives with systematic modifications of
542 charge and hydrophobicity (**Supplementary Information, chemistry**). All
543 synthesized compounds eliminated the stereocenter by replacing the cyclopentyl
544 group with a linear alkyl chain, thus simplifying the synthetic routes (Compounds

1 and 1-Est). Structural diversity was then introduced by adding a rigid motif, a diphenyl linker, on the flexible aliphatic chain bearing the acid moiety (Compounds 2 and 2-Est). Each compound was synthesized either with a free carboxylate or as ester to test the contribution of the anionic moiety (**Figure 5A**).

To rationalize differences in inhibition, we combined electrophysiology with molecular dynamics (MD) (**Figures S17 to S25**) and steered MD simulations of ligand unbinding (**Figures S30**). Five replicates per compound were simulated in LRRC8A:LRRC8C and LRRC8A:LRRC8E heteromers, totaling 25 μ s of simulation time. **DCPIB** and analogs retaining the carboxylic group remained stably bound, whereas ester derivatives consistently dissociated in all replicates. The acidic moiety established salt-bridge interactions predominantly with the R103 sidechains (interaction distances with R103: compound **1**, 3.93 ± 0.21 Å and compound **2**, 3.92 ± 0.15 Å). Unlike the other ester analogues, compound **2-Est** remained transiently bound at the selectivity filter due to the steric hindrance imposed by the biphenyl moiety, consistent with steric penalties limiting productive binding (**Figure 5B**).

PMF profiles confirmed these trends: acidic forms exhibited high unbinding barriers (**DCPIB** and Compound **1**: 14.4 ± 1.6 kcal/mol and 15.7 ± 1.1 kcal/mol respectively), while esters derivatives showed essentially barrierless processes (**DCPIB-Est** and Compound **1-Est**: -2.6 ± 2.0 kcal/mol and -3.4 ± 3.4 kcal/mol respectively), reflecting rapid dissociation. Biphenyl analogs increased PMF values (Compound **2** and **2-Est**: 30.3 ± 4.1 kcal/mol and 8.8 ± 4.6 kcal/mol respectively) but did not improve functional potency, since the bulky hydrophobic

568 effect of the biphenyl is likely to affect both binding and unbinding processes due
569 to steric hindrance (**Figure 5B**).

570 Electrophysiological assays mirrored the simulation results: ester
571 derivatives produced weaker block at equal concentrations, and displayed IC_{50}
572 shifts from $\sim 10 \mu M$ (DCPIB) to $>50 \mu M$ (**DCPIB-Est**) (**Figure 5C-D**). Despite
573 structural changes, all analogs adopted binding poses broadly similar to DCPIB
574 *in silico* (**Figure 5E**).

575 Together, these findings demonstrate that electrostatic complementarity,
576 particularly the presence of a carboxylate group, is essential for high-affinity
577 VRAC inhibition, whereas hydrophobic modifications modulate binding kinetics
578 without compensating for loss of charge. This establishes a mechanistic basis for
579 rational design of next-generation VRAC inhibitors.

580

581 Discussion

582 Our study provides mechanistic insights into DCPIB inhibition of VRAC
583 channels and underscores the challenges of defining inhibitor binding sites
584 through mutagenesis. Despite the strategic location of R103 in LRRC8A and its
585 critical role in ATP block, substitutions at this position (R103F, R103Q) did not
586 alter DCPIB sensitivity. This contrasts sharply with PANX2, where mutation of the
587 equivalent residue (R89) abolishes DCPIB inhibition (He et al., 2023) (**Figure 6**).
588 The divergent outcomes highlight fundamental differences in inhibitor recognition
589 between VRAC and pannexins, despite their shared electropositive ring at the
590 selectivity filter. In VRAC, DCPIB binding is highly adaptable, exploiting
591 alternative interactions with K51 and hydrophobic residues when R103 is absent,
592 as revealed by MD simulations. In PANX2, by contrast, the rigid extracellular
593 architecture and lack of compensatory contacts likely render R89 indispensable
594 for inhibitor binding.

595 Attempts to abolish DCPIB binding in VRAC by targeting compensatory
596 residues were unsuccessful. Although K51 was predicted to contribute to the
597 secondary binding pose observed in R103F mutants, the combined K51F-R103F
598 substitution rendered the channel non-functional, precluding pharmacological
599 assessment. Both R103 and K51 establish a strong electrostatic potential that
600 guides anions through VRAC, making them essential for chloride selectivity and
601 the channel function. Sequence alignment further reveals that PANX2 lacks a
602 positively charged residue analogous to K51 (E77 in PANX2, **Figure 6**),
603 suggesting that VRAC relies on a more distributed interaction network that
604 enables inhibitor binding plasticity. These structural differences help explain why

605 mutagenesis can define the binding site in PANX2 but not in VRAC, where DCPIB
606 engages multiple residues through compensatory interactions.

607 The design and evaluation of DCPIB analogs reinforce the central role of
608 electrostatics in VRAC inhibition. MD and steered MD simulations demonstrated
609 that analogs retaining the carboxylate group exhibit binding stability and
610 unbinding barriers comparable to DCPIB, whereas ester derivatives display rapid
611 dissociation and markedly reduced potency. Electrophysiological assays
612 confirmed this trend, with IC_{50} values shifting from $\sim 10 \mu M$ for DCPIB to $>50 \mu M$
613 for its ester form. Together, these observations indicate that charge
614 complementarity at the selectivity filter is a primary determinant of high-affinity
615 inhibition, whereas hydrophobic modifications primarily modulate binding kinetics
616 without compensating for loss of electrostatic interactions.

617 Our findings complement recent work by Yamada et al. (2025), who
618 identified a conserved mechanism of VRAC inhibition by two structurally distinct
619 lipophilic drugs, zafirlukast and pranlukast (Yamada et al., 2025). Using cryo-EM-
620 guided modeling and mutagenesis, they showed that these compounds bind
621 within transmembrane fenestrations, displace pore-associated lipids (Takahashi
622 et al., 2023), and promote voltage-dependent inactivation. Interestingly, the
623 correlation between inhibitor potency and inactivation parallels our observation
624 that DCPIB binding remains robust despite mutations in the selectivity filter,
625 suggesting that VRAC inhibition can arise from multiple, partially independent
626 structural determinants, including electrostatic complementarity and lipid
627 displacement. Together, these studies underscore the complexity of VRAC

628 pharmacology and highlight lipid–channel interactions as a common theme in the
629 action of highly lipophilic inhibitors such as DCPIB, zafirlukast, and pranlukast.

630 Taken together, these findings highlight two major challenges in VRAC
631 pharmacology: the scarcity of potent and selective inhibitors and the difficulty of
632 validating binding mechanisms through mutagenesis. The structural similarity
633 between VRAC, PANX1 and PANX2, coupled with their shared sensitivity to CBX
634 or DCPIB (**Figure 6**), emphasizes the need for rational optimization of inhibitor
635 chemistry, preserving anionic moieties while exploring scaffold modifications, to
636 achieve selectivity and potency. Future progress will require high-resolution
637 structural studies of VRAC-inhibitor complexes, combined with computational
638 and chemical approaches, to advance the development of next-generation VRAC
639 modulators.

640

641 **Data availability**

642 The datasets generated during the current study are available from the
643 corresponding author on reasonable request.

644

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901 **Legend of Figures**

902 **Figure 1. Effect of R103F mutation on ATP and DCPIB inhibition of VRAC**
903 **currents in *LRRC8*^{-/-} HEK293T cells.**

904 Whole-cell patch-clamp recordings were performed in *LRRC8*^{-/-} HEK293T cells
905 co-expressing LRRC8E together with either wild-type LRRC8A or LRRC8A
906 (R103F) using bicistronic T2A constructs. Currents were elicited under hypotonic
907 conditions to activate VRAC.

908 For clarity, the traces shown are normalized and displayed around inhibitor
909 application. The hypotonic solution was applied 3-4 min before ATP or DCPIB
910 application, to ensure high resolution VRAC currents activation. Baseline currents
911 under isotonic conditions were close to zero and are not shown in the normalized
912 traces. **(A)** Left, normalized time course showing inhibition by 2 mM ATP at ± 75
913 mV. Right, summary bar graphs showing percentage inhibition for ATP (mean $\pm$
914 SD; n = 5 WT, n = 9 R103F). **(B)** Left, normalized time course during application
915 of 10 μ M DCPIB at ± 75 mV. Right, summary bar graphs showing percentage
916 inhibition for DCPIB (mean $\pm$ SD; n = 5 WT, n = 5 R103F). Statistical significance
917 was assessed using two-tailed t-test: ***p < 0.001 for ATP inhibition (WT vs
918 R103F); ns, not significant for DCPIB.

919

920 **Figure 2. Functional analysis of LRRC8A and LRRC8E mutants in *Xenopus***

921 **oocytes: sensitivity to ATP, DCPIB and CBX.** Two-electrode voltage clamp
922 recordings were performed under isotonic conditions in *Xenopus* oocytes co-
923 expressing LRRC8A-VFP (WT or mutants) and LRRC8E-mCh (WT or mutants).
924 **(A)** Representative current traces for WT, R103F and R103Q channels during
925 voltage steps from -90 mV to +70 mV. **(B)** Bar graphs summarizing inhibition by

926 DCPIB (20 μ M) at +60 mV for WT, R103F, and R103Q channels (mean $\pm$ SD; n
 927 = 8-13; two-tailed t-test). **(C)** Bar graphs summarizing inhibition by ATP (2 mM)
 928 at +60 mV for WT and R103F channels (mean $\pm$ SD; p value is indicated in the
 929 figure, WT vs R103F, n = 3, two-tailed t-test). **(D)** Concentration–response curves
 930 for CBX inhibition in WT and R103F channels (WT IC₅₀ = 12.04 μ M, Hill = 1.45,
 931 n = 4; R103F IC₅₀ = 11.43 μ M, Hill = 1.63, n = 4). **(E)** Representative traces from
 932 oocytes co-expressing LRRC8A-VFP with LRRC8E-mCh mutants (L96A or
 933 Q97E). **(F)** Bar graphs summarizing inhibition by DCPIB (20 μ M) for WT and
 934 LRRC8E mutants (L96A, Q97E) (mean $\pm$ SD; p value is indicated in the figure,
 935 WT vs Q97E, n = 6-14; one-way ANOVA followed by Tukey's post hoc). **(G)**
 936 Concentration–response curves for DCPIB inhibition in WT and Q97E (WT = 8.59
 937 $\pm$ 0.38 μ M; Q97E = 10.95 $\pm$ 2.37 μ M; ns, two-tailed t-test; n = 4–5 oocytes per
 938 condition).

939

940 **Figure 3. Binding modes of ATP and DCPIB in LRRC8 channels revealed by**
 941 **molecular modeling.** Structural models of LRRC8 heteromers were generated
 942 based on cryo-EM templates and used for molecular dynamics simulations to
 943 visualize ligand binding at the selectivity filter. **(A)** Model of the transmembrane
 944 domain showing DCPIB bound at the selectivity filter. Orientation: extracellular
 945 (EC) and intracellular (IC) sides indicated. **(B)** Binding mode of the ATP in the
 946 WT channel, forming salt bridges with R103 and contacts with D104 and L105.
 947 **(C, D)** Two alternative binding poses of DCPIB inhibitor in the WT
 948 LRRC8A:LRRC8C channel: one interacting with R103 and another shifted inward
 949 toward K51. **(E)** Binding mode of DCPIB in the R103F mutant LRRC8A:LRRC8C
 950 channel, showing π -stacking with F103 and compensatory interactions with K51.

951 Wildtype LRRC8A and LRRC8C monomers are shown in blue and grey cartoons;
952 the R103F LRRC8A mutant is shown in green. ATP and DCPIB are represented
953 as yellow and orange sticks, respectively. Two LRRC8A monomers are omitted
954 for clarity.

955

956 **Figure 4. Impact of selectivity filter mutations on pore electrostatics, ion**
957 **occupancy and DCPIB binding. (A)** Localization of residues targeted for
958 mutagenesis (R103, K51, T48) within the LRRC8A monomer structure. **(B)**
959 Electrostatic potential maps of the pore for WT and mutant channels (R103F,
960 R103F-K51F, T48D) calculated using APBS. Color scale is shown in kcal/mol.
961 **(C)** Isodensity maps from equilibrium MD simulations showing chloride (green)
962 and potassium (purple) densities in the inner vestibule for WT and mutant
963 channels in the apo state. Isodensity value = 0.025 g/cm³. **(D)** Left, representative
964 current traces recorded in *Xenopus laevis* oocytes expressing WT channels or
965 channels carrying single K51 substitutions (K51Q in LRRC8A or LRRC8E),
966 illustrating preserved channel function. Right, summary bar graph showing
967 inhibition by DCPIB (20 μM) for WT and K51 mutant channels (mean ± SD; *n* = 3).
968 **(E)** Representative current traces for uninjected oocytes and oocytes expressing
969 the K51F-R103F double mutant, illustrating loss of detectable channel activity.
970 **(F)** Two alternative binding poses of DCPIB inhibitor within the selectivity filter of
971 the T48D mutant channel obtained from MD simulations, showing interaction
972 mediated by R103 and K51 side chains. **(G)** Left, representative current traces
973 for the T48D mutant recorded at +60 mV under isotonic conditions. Right,
974 summary bar graph showing inhibition by DCPIB (20 μM) for WT and T48D

975 channels (mean $\pm$ SD; n = 7). Statistical significance was assessed using a two-
976 tailed *t*-test.

977

978 **Figure 5. Analysis of DCPIB analogues by MD and electrophysiology (A)**

979 Chemical structures of **DCPIB** and synthesized analogues (**DCPIB-Est**, **1**, **1-Est**,
980 **2**, **2-Est**). Structural modifications include introduction of a diphenyl motif
981 (compound 2), replacement of the cyclopentyl group by a linear C4 chain, and
982 presentation of the terminal carbonyl as carboxylate or ethyl/methyl ester. **(B)**
983 Steered MD unbinding profiles (PMF) profiles for DCPIB and analogues in
984 LRRC8A:LRRC8E channels. The collective variable is the COM distance of the
985 indanone scaffold to the selectivity filter (T48/M48). Curves show mean $\pm$
986 confidence interval obtained by bootstrapping of 100 replicates. **(C)** Bar graphs
987 summarizing inhibition at 20 μ M for DCPIB and analogues in *Xenopus* oocytes
988 expressing LRRC8A/8E. (mean $\pm$ SD; n = 4 -17). P-values in the figure in black
989 indicate the statistical significance comparing acid vs ester forms; P-values in the
990 figure in red indicate the statistical significance relative to DCPIB (one-way
991 ANOVA followed by Tukey's post hoc). **(D)** Dose-response curves comparing
992 DCPIB and its ester counterpart (DCPIB-Est). Normalized current (I_{inhib}/I_0) plotted
993 versus [inhibitor] (μ M); fits to a four-parameter Hill model (IC_{50} and Hill slope
994 reported on the text). **(E)** Representative binding poses from equilibrium MD for
995 selected analogues in the selectivity filter of LRRC8 heteromers, highlighting
996 contacts with R103, K51 and hydrophobic residues (L105 in LRRC8C; L96/Q97
997 in LRRC8E). DCPIB is shown as grey sticks and its analogues 1, 2 and 2-Est are
998 shown as blue, orange and maroon sticks respectively; LRRC8A and partner

999 subunits (8C or 8E) as blue and gray cartoons; key residues labeled. Two
1000 LRRC8A subunits are omitted for clarity.

1001

1002 **Figure 6. Structural comparison of the extracellular selectivity filter in**
1003 **LRRC8A and human pannexins. (A) LRRC8A structure** (PDB ID: 6NZW)
1004 showing the selectivity filter and the binding mode of DCPIB. **(B)** Human pannexin
1005 1 (PANX1; PDB ID: 6LTO) and **(C)** Human pannexin 2 (PANX2; PDB ID: 7XLB).
1006 Key residues shaping the selectivity filter in LRRC8A (R103, K51, T48) and their
1007 pannexin equivalents are shown in sticks and labeled. Proteins are represented
1008 as transparent surface and cartoon for clarity; DCPIB is shown in orange sticks
1009 in LRRC8A.

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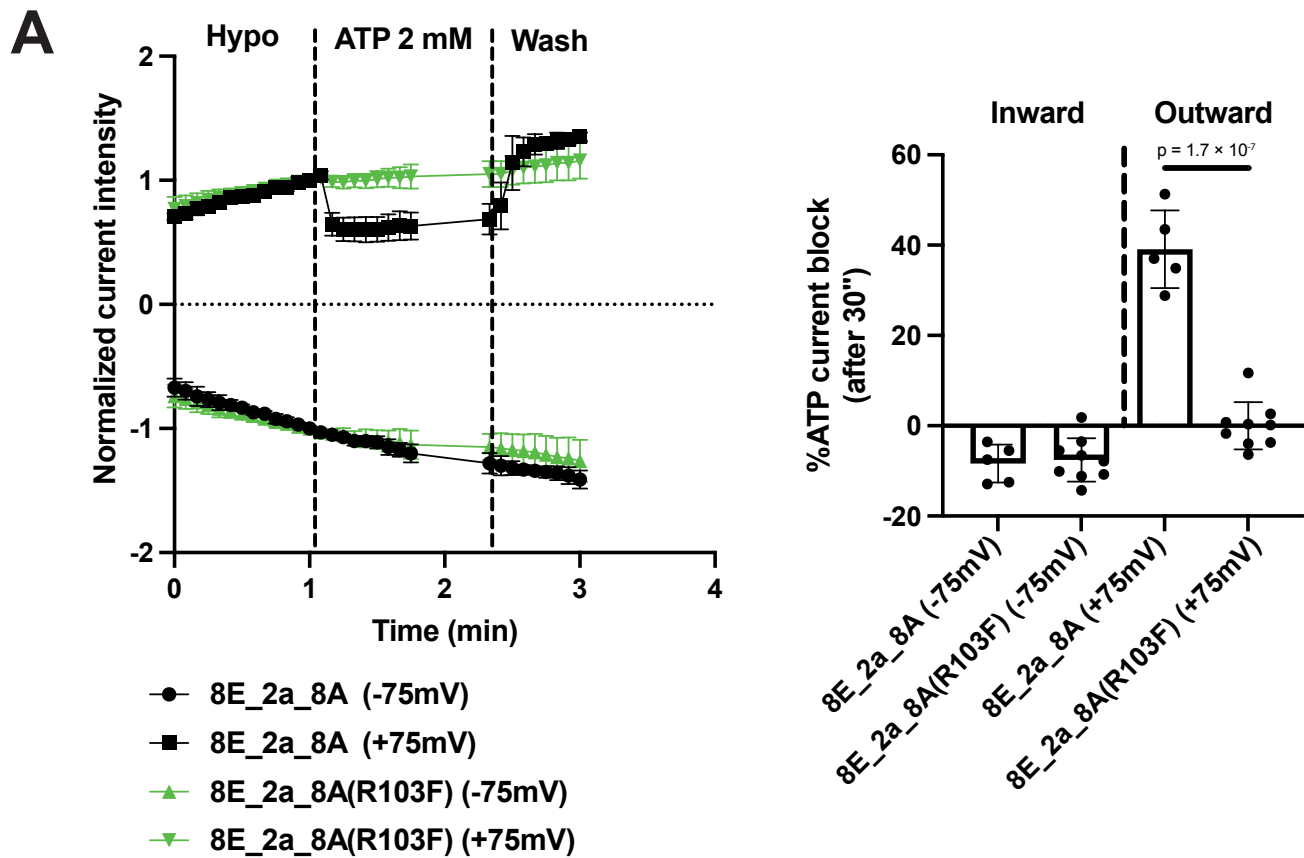
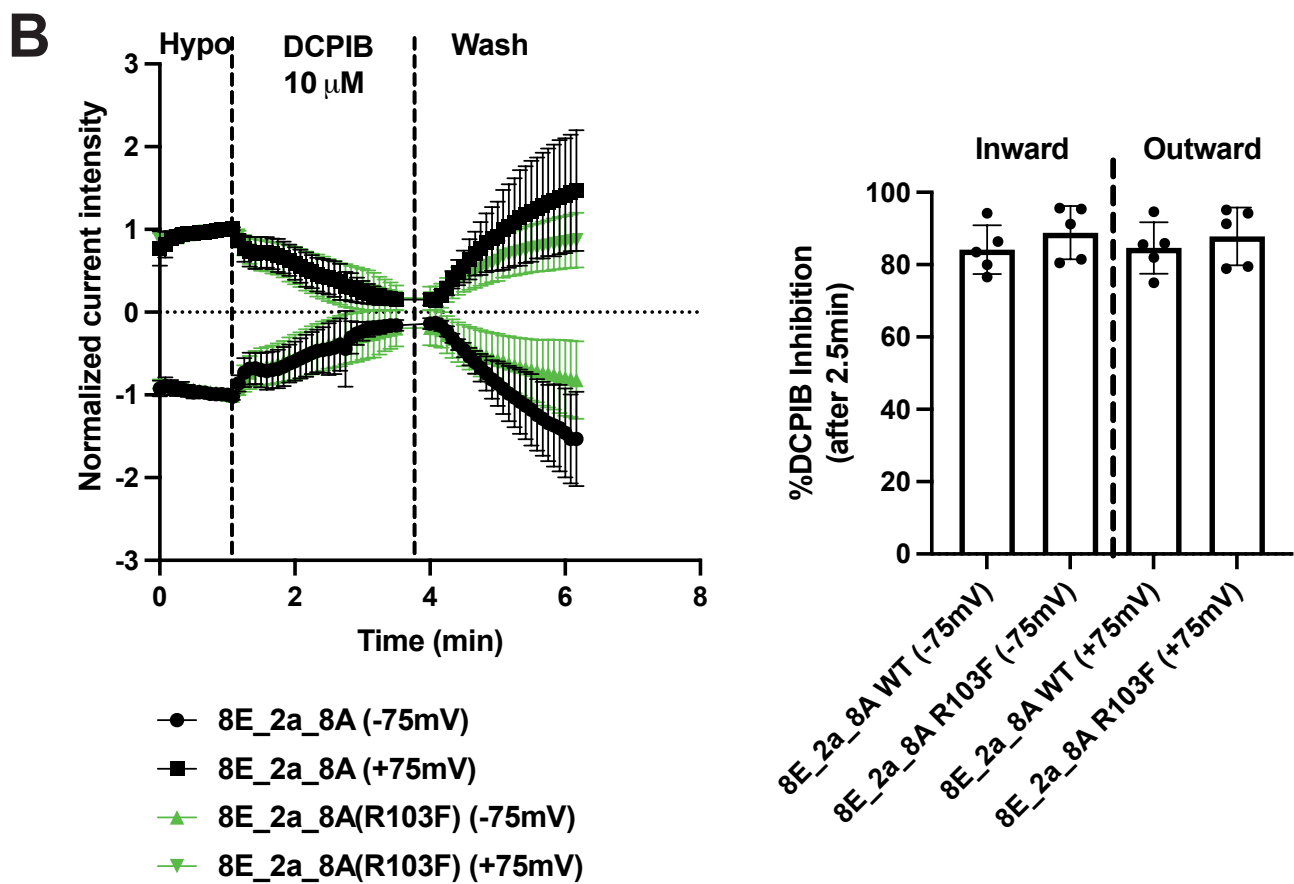


Figure 1

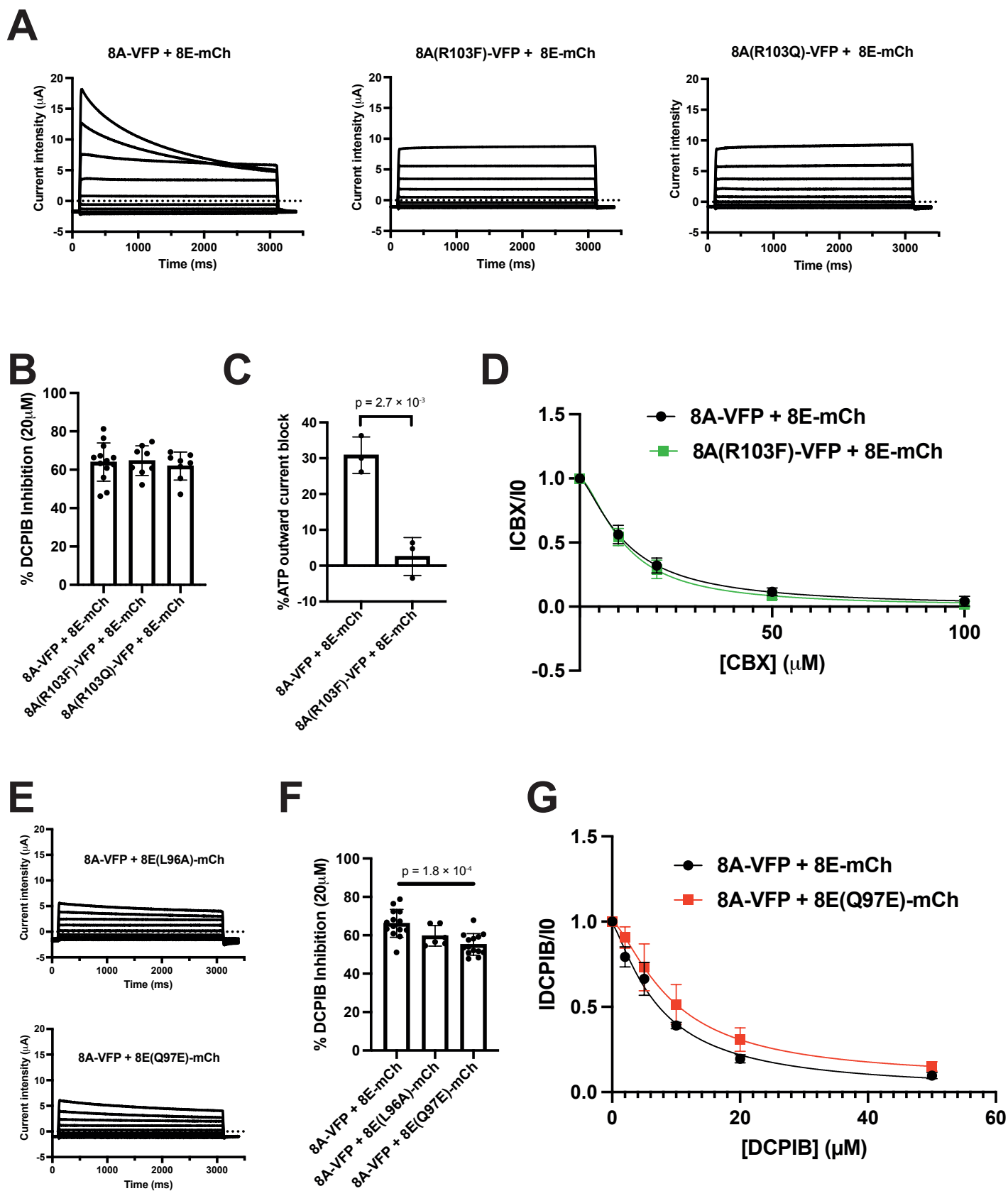


Figure 2

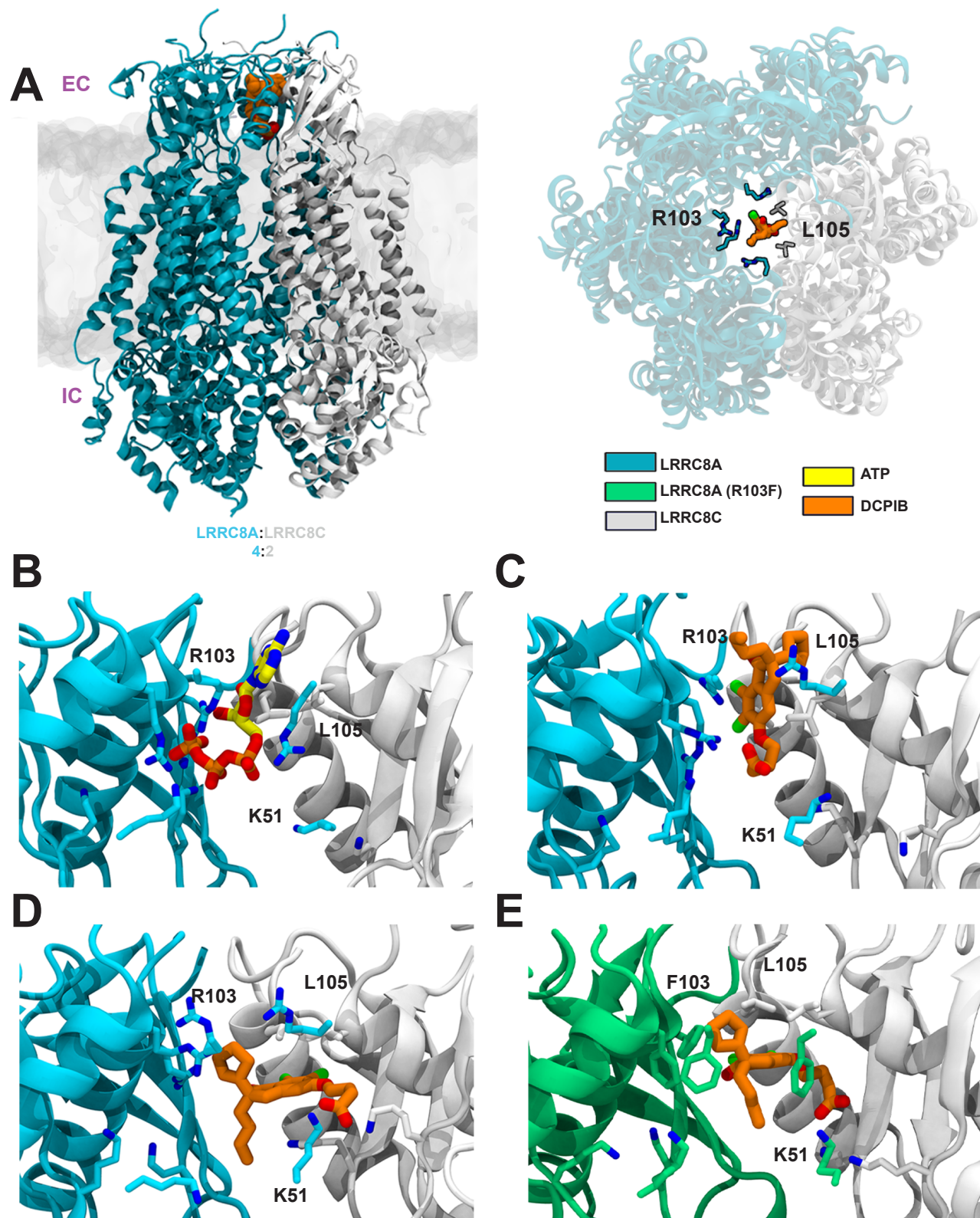


Figure 3

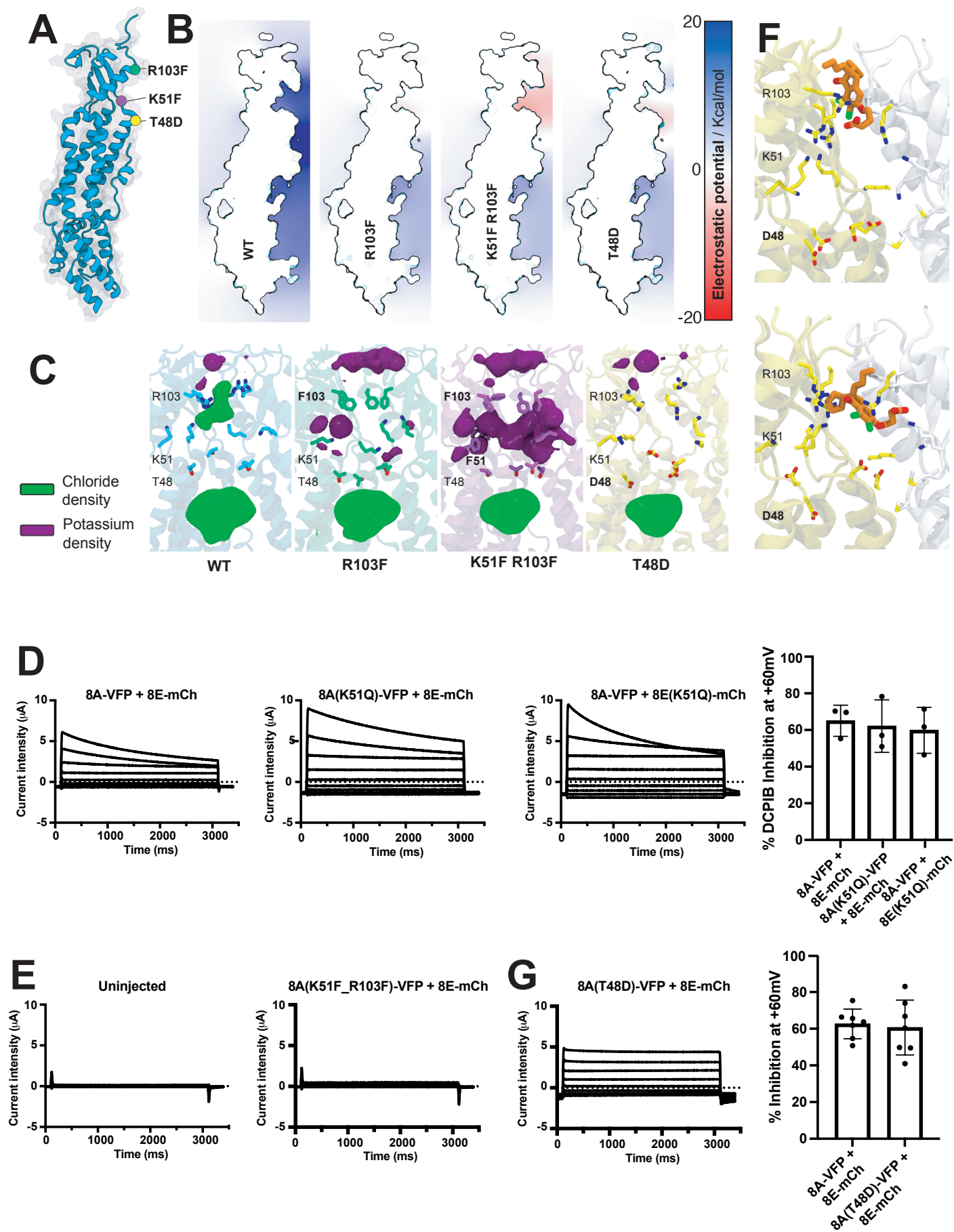
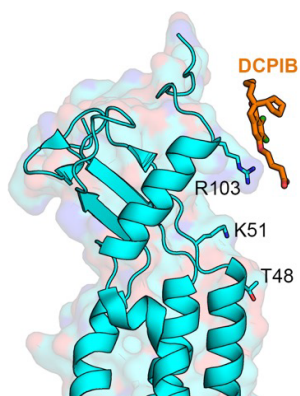
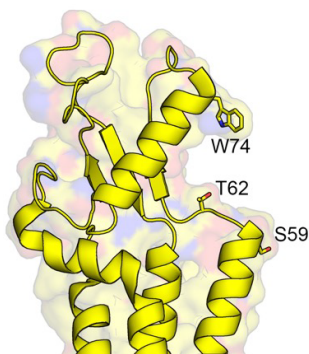


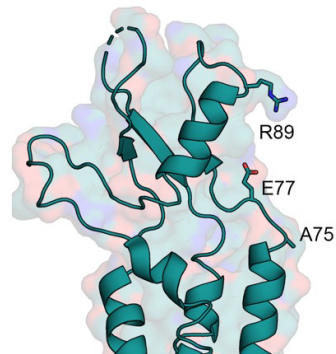
Figure 4



Human LRRC8A
PDB id: 6NZW



Human Pannexin 1
PDB id: 6LTO



Human Pannexin 2
PDB id: 7XLB

Figure 6

Supporting information

Index

Guide to the Supporting Information

This Supporting Information is organized into three main types of analyses:

(i) Structural stability of the simulated systems, assessed by root-mean-squared deviation (RMSD) (Figures S3–S27).

(ii) Ligand–protein interactions, quantified as minimum distances between key residues in the selectivity filter (Figures S4–S7, S10, S14, S16, S18–S27).

(iii) Binding mode analysis, including distance histograms and quantitative summaries of interaction patterns (Figures S28–S29 and Table S3).

For clarity:

- RMSD plots report the structural stability of the protein and ligand throughout the simulations.
- Distance plots describe interactions between ligand functional groups and residues R103 and K51 (or their corresponding mutants).
- Histograms (Figure S28) and Table S3 provide a quantitative description of binding modes across all simulated systems.

Figure S1. Overview of the computational models included in this work.

Figure S2. Comparison of the density of the DCPIB between CryoEM and Molecular Dynamics simulations.

Figure S3. Structural analysis of the LRRC8A:LRRC8C (4:2) channel.

Figure S4. Structural analysis of the complex ATP - LRRC8A:LRRC8C (4:2) channel.

Figure S5. Structural analysis of the complex ATP - LRRC8A:LRRC8E (4:2) channel.

Figure S6. Structural analysis of the complex DCPIB - LRRC8A:LRRC8C (4:2) channel.

Figure S7. Structural analysis of the complex DCPIB - LRRC8A:LRRC8E (4:2) channel.

Figure S8. Structural analysis of the *R103F* LRRC8A:LRRC8C (4:2) channel.

Figure S9. Structural analysis of the complex ATP – *R103F* LRRC8A:LRRC8C (4:2) channel.

Figure S10. Structural analysis of the complex DCPIB – *R103F* LRRC8A:LRRC8C (4:2) channel.

Figure S11. Structural analysis of the *K51F R103F* LRRC8A:LRRC8C (4:2) channel.

Figure S12. Structural analysis of the complex DCPIB – *K51F R103F* LRRC8A:LRRC8C (4:2) channel.

Figure S13. Structural analysis of the *T48D* LRRC8A:LRRC8C (4:2) channel.

Figure S14. Structural analysis of the complex *T48D* DCPIB - LRRC8A:LRRC8C (4:2) channel.

Figure S15. Structural analysis of the *K51Q* LRRC8A:LRRC8C (4:2) channel.

Figure S16. Structural analysis of the complex *K51Q* DCPIB - LRRC8A:LRRC8C (4:2) channel.

Figure S17. Structural analysis of the complex DCPIB-Est - LRRC8A:LRRC8C (4:2) channel.

Figure S18. Structural analysis of the complex 1 - LRRC8A:LRRC8C (4:2) channel.

Figure S19. Structural analysis of the complex 1 - LRRC8A:LRRC8E (4:2) channel.

Figure S20. Structural analysis of the complex 1-Est - LRRC8A:LRRC8C (4:2) channel.

Figure S21. Structural analysis of the complex 1-Est - LRRC8A:LRRC8E (4:2) channel.

Figure S22. Structural analysis of the complex 2 - LRRC8A:LRRC8C (4:2) channel

Figure S23. Structural analysis of the complex 2 - LRRC8A:LRRC8E (4:2) channel.

Figure S24. Structural analysis of the complex 2-Est - LRRC8A:LRRC8C (4:2) channel.

Figure S25. Structural analysis of the complex 2-Est - LRRC8A:LRRC8E (4:2) channel.

Figure S26. Structural analysis of the complex DCPIB - LRRC8A channel.

Figure S27. Structural analysis of the complex DCPIB - LRRC8A channel.

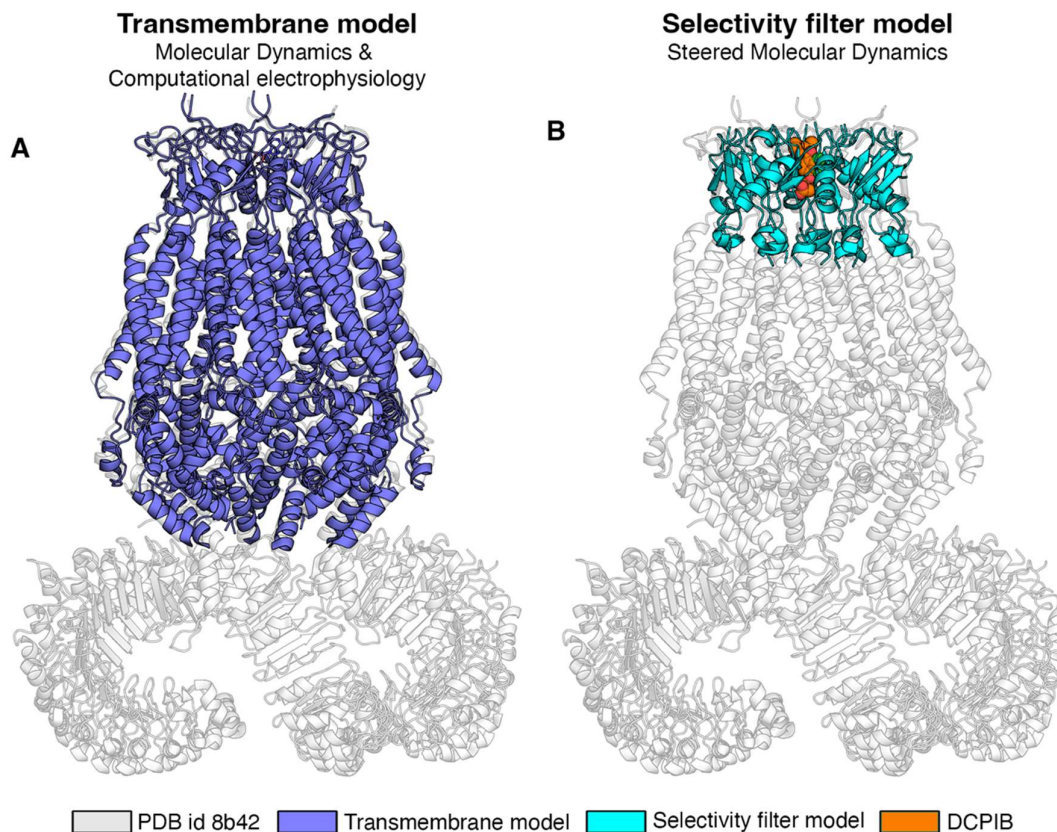
Figure S28. Histograms of the interaction distances between DCPIB and its analogues and the LRRC8A:LRRC8C (4:2) and LRRC8A:LRRC8E (4:2) channels.

Figure S29. Visualization of DCPIB binding modes and its interaction distances with the R103 and K51 sidechains.

Figure S30. Computational details of the Steered molecular dynamics simulations.

Table S1. Membrane composition of the transmembrane model.

63 **Table S2.** Summary table of the molecular dynamics simulations.
64 **Table S3.** Table detailing percentages of each binding mode for each system described in this work.
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75 **Figure S1. Overview of the computational models included in this work. (A)** Overlap of the full
 76 length VRAC channel (PDB id 8b42) and the model that only includes the transmembrane domain used
 77 for the molecular dynamics simulations and the computational electrophysiology simulations. **(B)**
 78 Overlap of the full length VRAC channel (PDB id 8b42) and the model that only includes the selectivity
 79 filter used for the steered molecular dynamics simulations.

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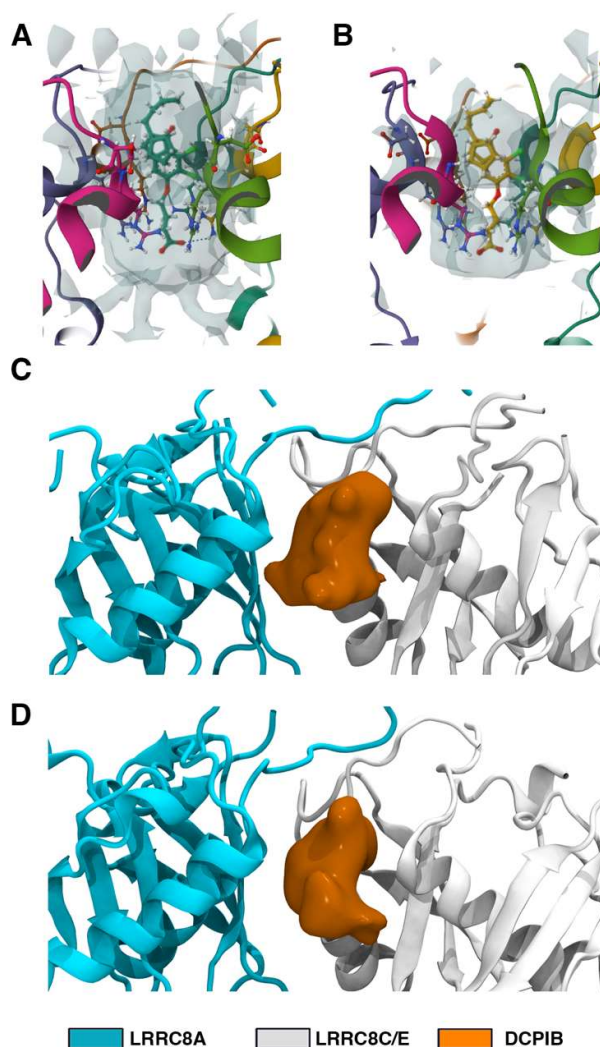


Figure S2. Comparison of the density of the DCPIB between CryoEM and Molecular Dynamics simulations. **(A)** Density of the DCPIB in the selectivity filter from the Cryo-EM structure (PDB id 6NZW, 3.21Å) as shown in the PDB. **(B)** Density of the DCPIB in the selectivity filter from the Cryo-EM structure (PDB id 6NZZ, 3.32Å) as shown in the PDB. **(C)** The densities of the DCPIB calculated from the molecular dynamics simulations of the DCPIB bound LRRC8A:LRRC8C system. **(D)** The densities of the DCPIB calculated from the molecular dynamics simulations of the DCPIB bound LRRC8A:LRRC8C system. The orange isosurfaces accounts for 20% of density of the DCPIB during all the simulation time (aggregated 2500 ns of simulation time).

LRRC8A:LRRC8C (4:2) complex

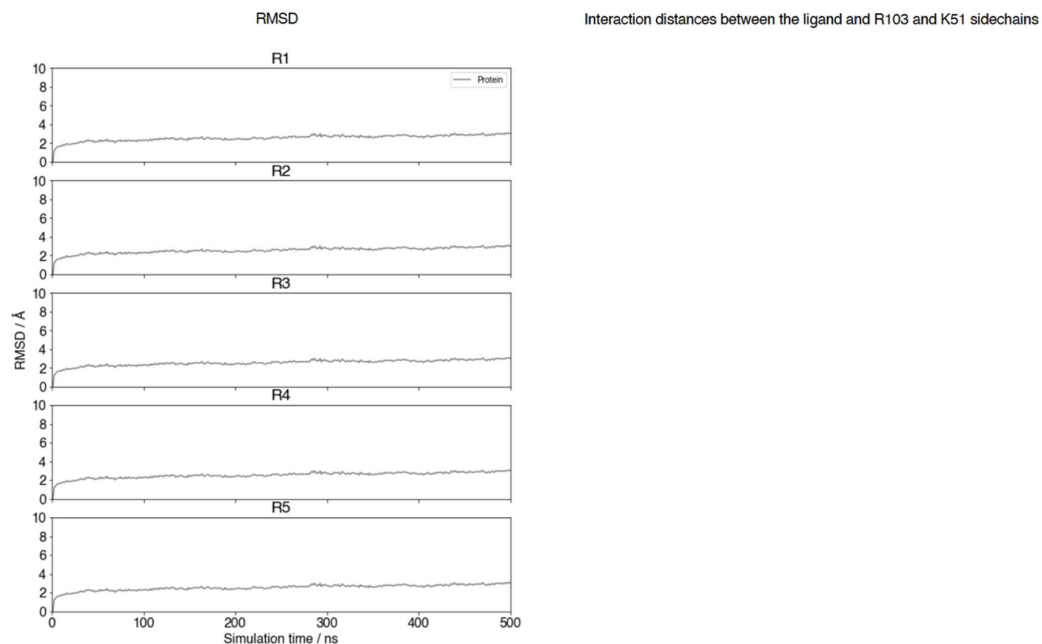


Figure S3. Structural analysis of the LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: the Ca atoms of the protein are shown as grey lines. These data indicate stable equilibration of the system after the initial phase of the simulations.

ATP - LRR8A:LRR8C (4:2) complex

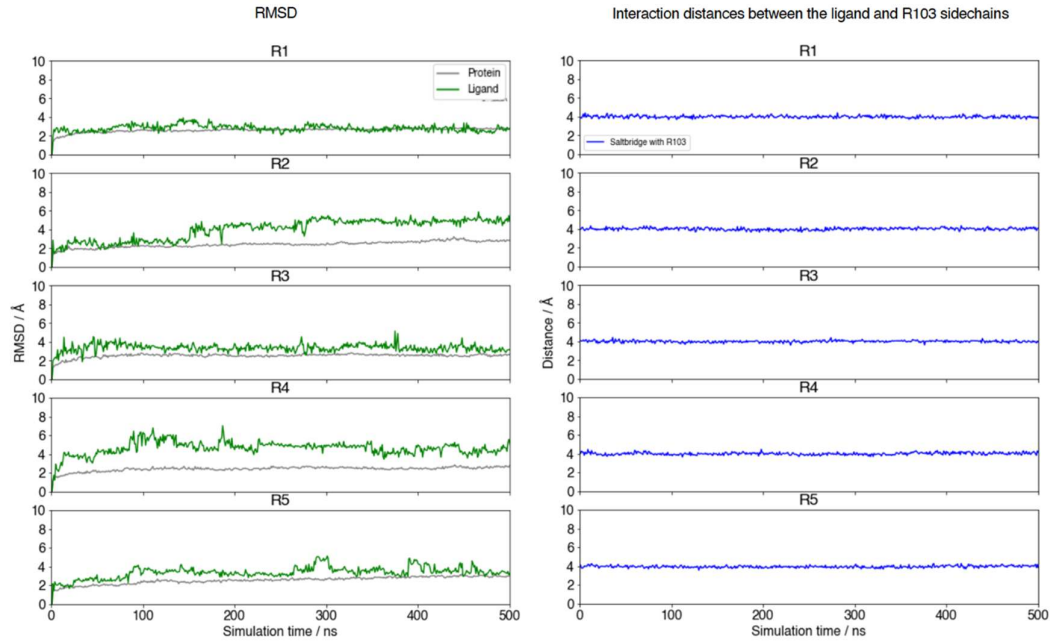


Figure S4. Structural analysis of the complex ATP - LRR8A:LRR8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the phosphate groups of ATP and the atom C_z of the R103 sidechains (Å) along the simulations time shown as blue lines. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

ATP - LRR8A:LRR8E (4:2) complex

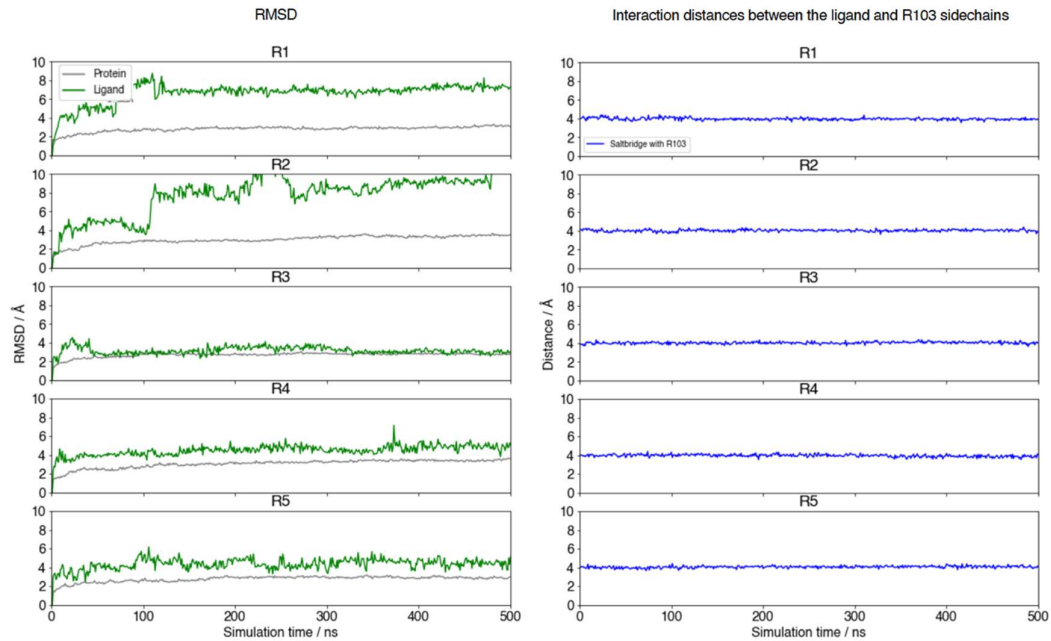


Figure S5. Structural analysis of the complex ATP - LRR8A:LRR8E (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the phosphate groups of ATP and the atom C_z of the R103 sidechains (Å) along the simulations time shown as blue lines. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

DCPIB - LRRC8A:LRRC8C (4:2) complex

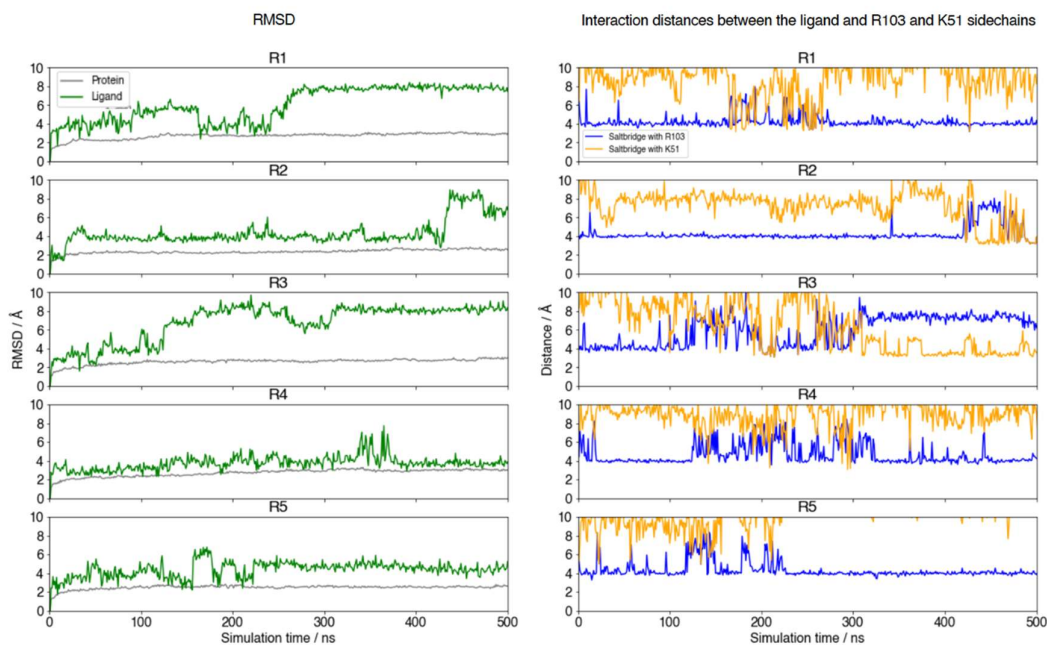


Figure S6. Structural analysis of the complex DCPIB - LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

DCPIB - LRRC8A:LRRC8E (4:2) complex

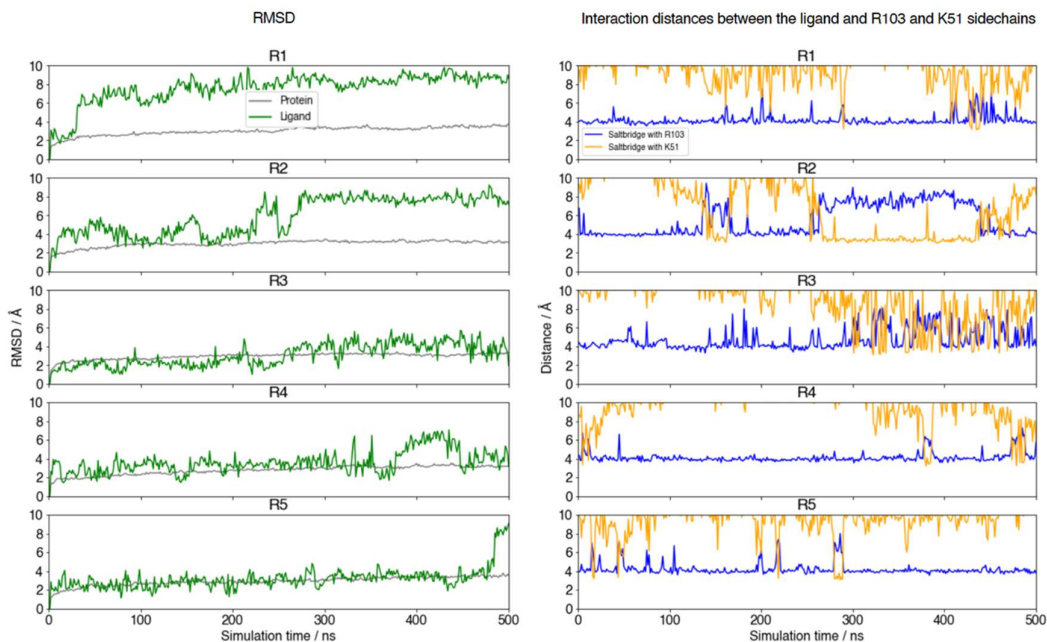


Figure S7. Structural analysis of the complex DCPIB - LRRC8A:LRRC8E (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

R103F LRRC8A:LRRC8C (4:2) complex

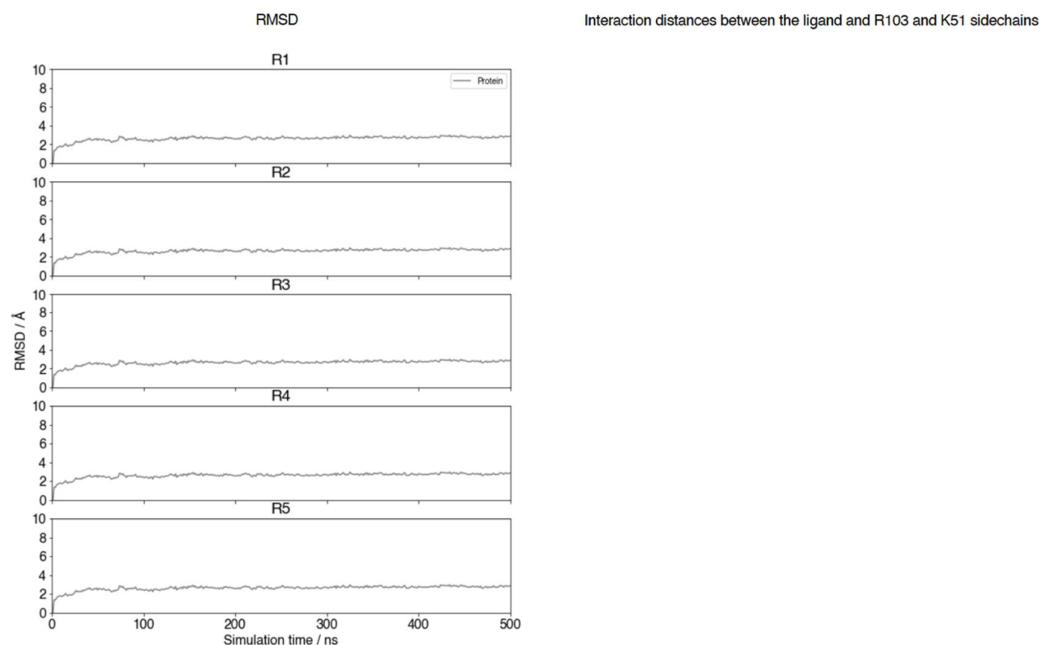


Figure S8. Structural analysis of the *R103F* LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: the Ca atoms of the protein are shown as grey lines. **These data indicate stable equilibration of the system after the initial phase of the simulations.**

ATP – *R103F* LRRC8A:LRRC8C (4:2) complex

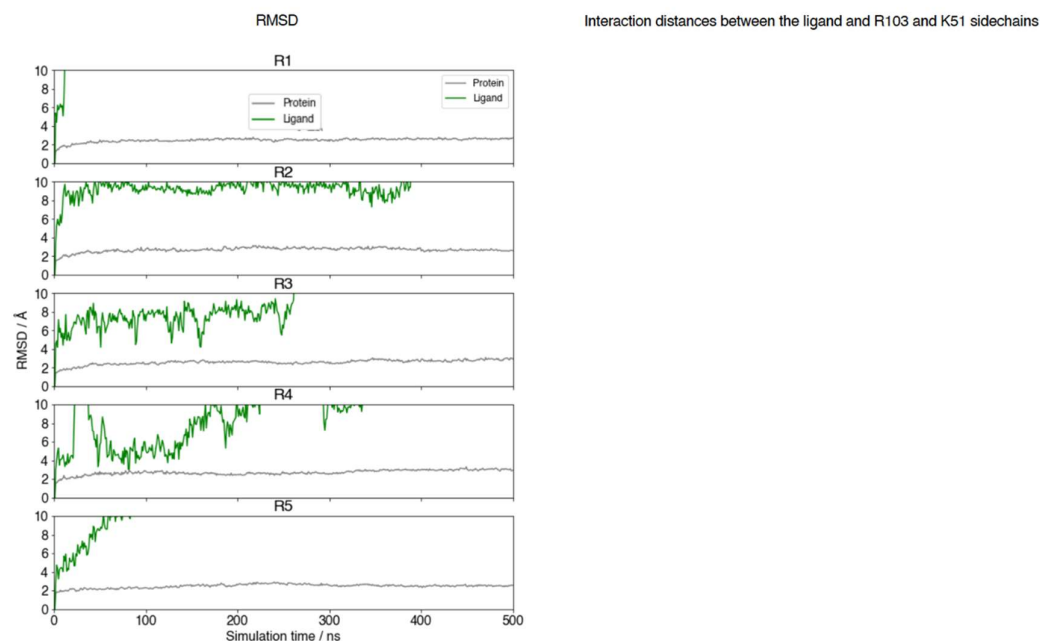


Figure S9. Structural analysis of the complex ATP – *R103F* LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. **These data indicate stable equilibration of the system after the initial phase of the simulations.**

DCPIB – R103F LRRC8A:LRRC8C (4:2) complex

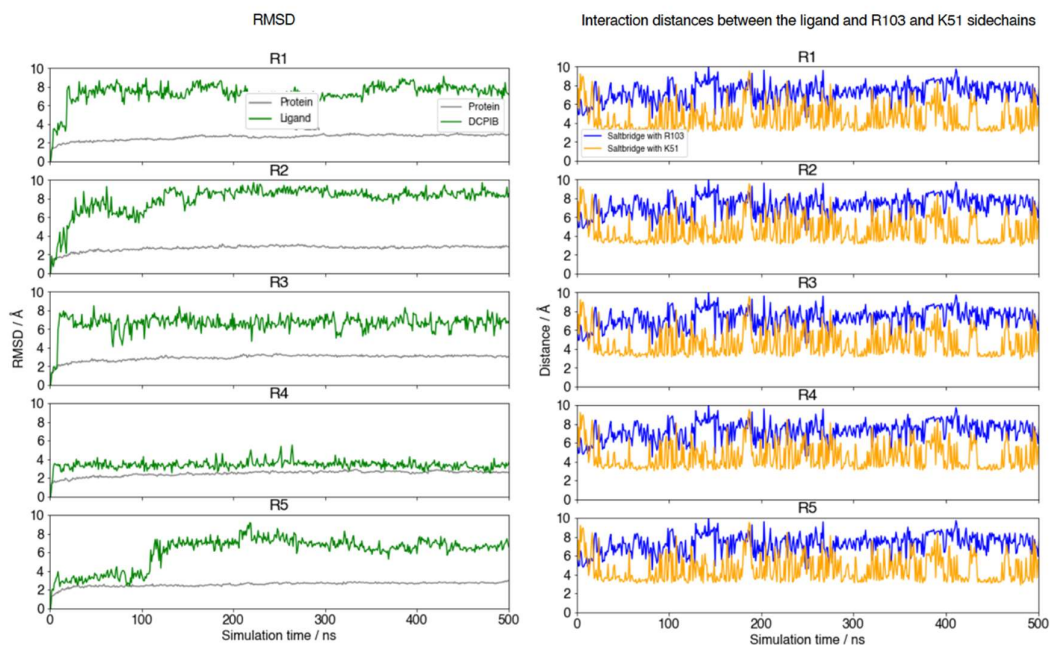


Figure S10. Structural analysis of the complex DCPIB – R103F LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the F103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. **These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.**

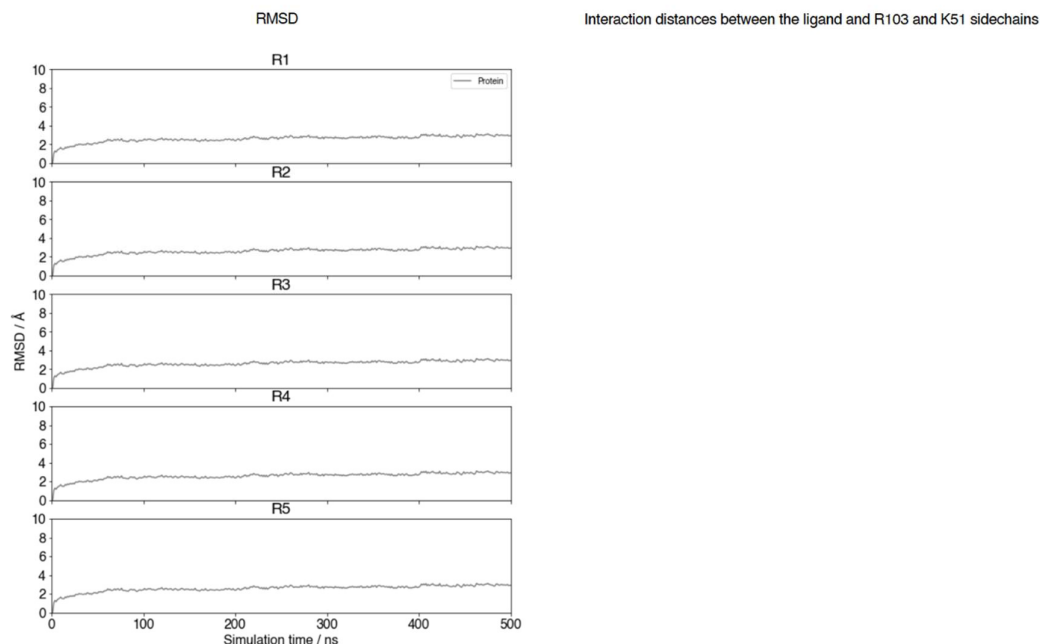
***K51F R103F* LRRC8A:LRRC8C (4:2) complex**

Figure S11. Structural analysis of the *K51F R103F* LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: the Ca atoms of the protein are shown as grey lines. These data indicate stable equilibration of the system after the initial phase of the simulations.

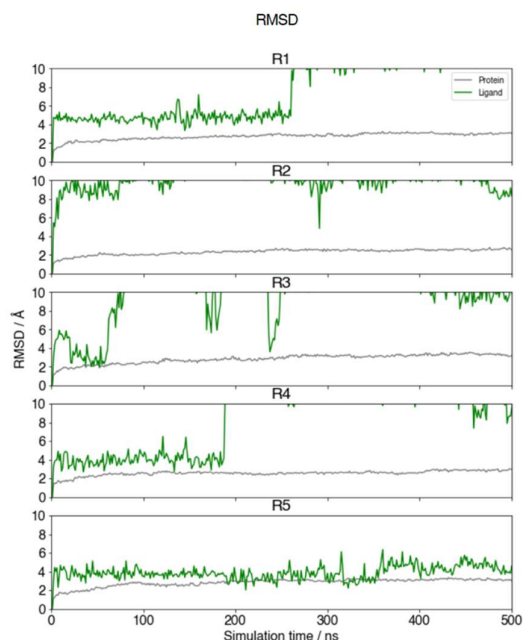
DCPIB – *K51F R103F* LRRC8A:LRRC8C (4:2) complex

Figure S12. Structural analysis of the complex DCPIB – *K51F R103F* LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. These data indicate stable equilibration of the system after the initial phase of the simulations.

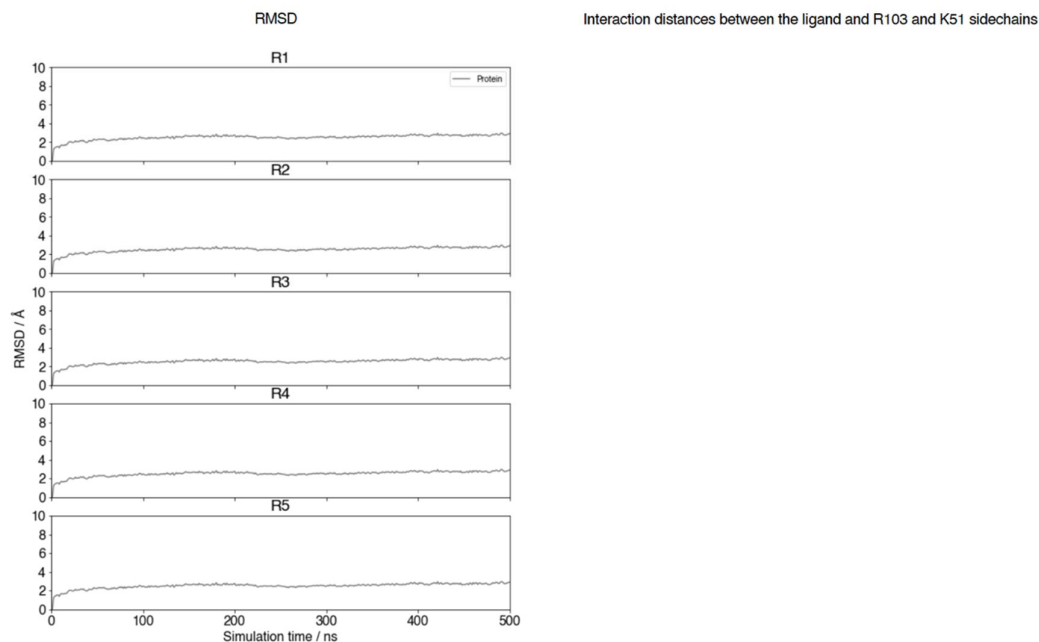
***T48D* LRRC8A:LRRC8C (4:2) complex**

Figure S13. Structural analysis of the *T48D* LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: the Ca atoms of the protein are shown as grey lines. These data indicate stable equilibration of the system after the initial phase of the simulations.

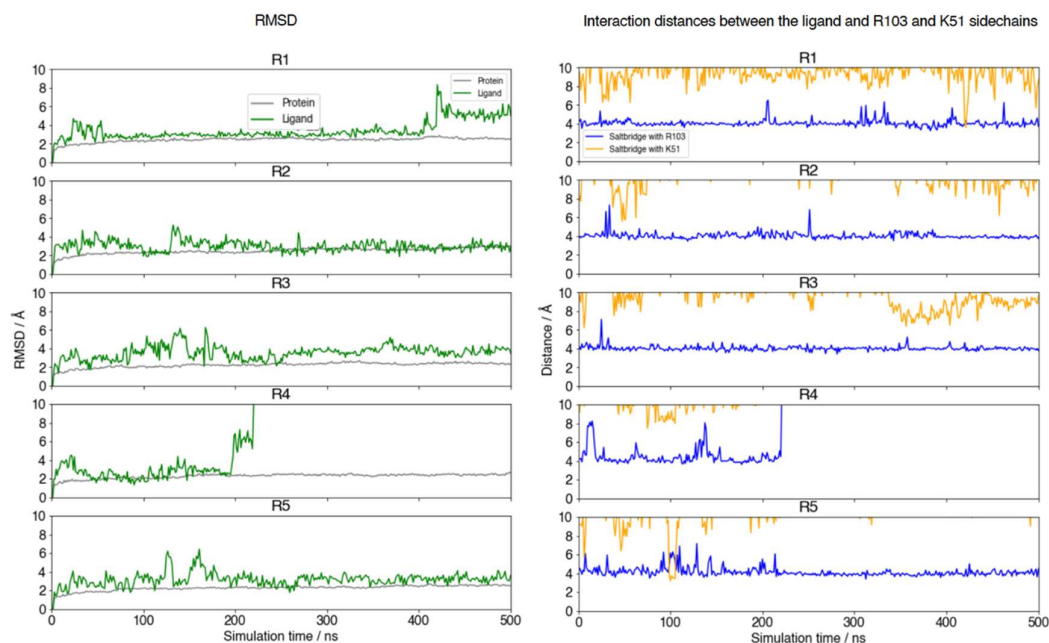
DCPIB – *T48D* LRRC8A:LRRC8C (4:2) complex

Figure S14. Structural analysis of the complex *T48D* DCPIB - LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

K51Q LRRC8A:LRRC8C (4:2) complex

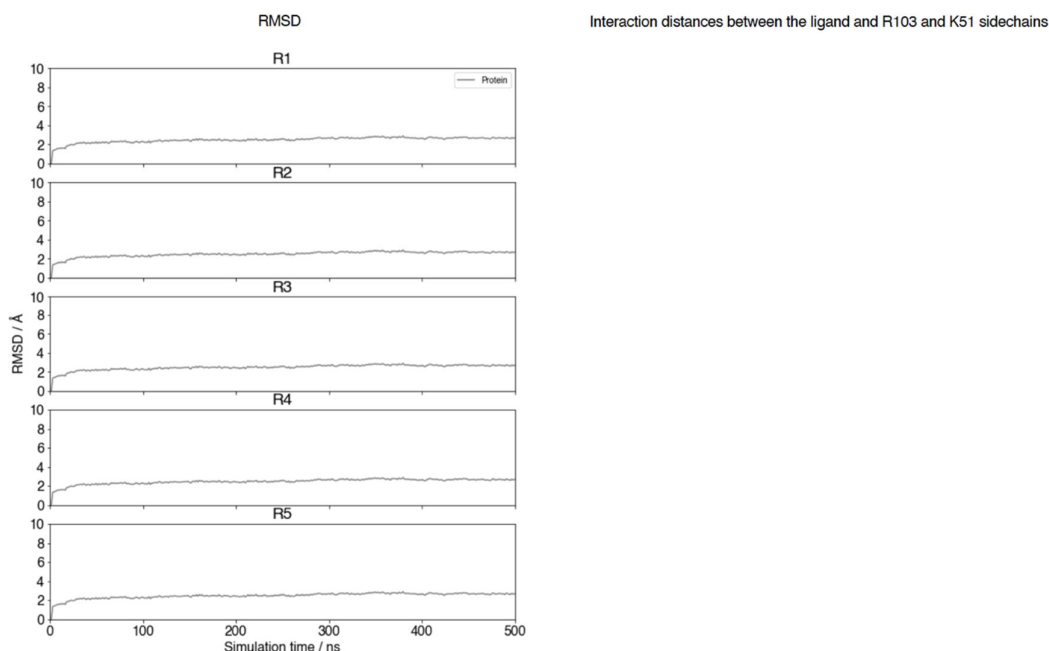


Figure S15. Structural analysis of the K51Q LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: the Ca atoms of the protein are shown as grey lines. **These data indicate stable equilibration of the system after the initial phase of the simulations.**

DCPIB - K51Q LRRC8A:LRRC8C (4:2) complex

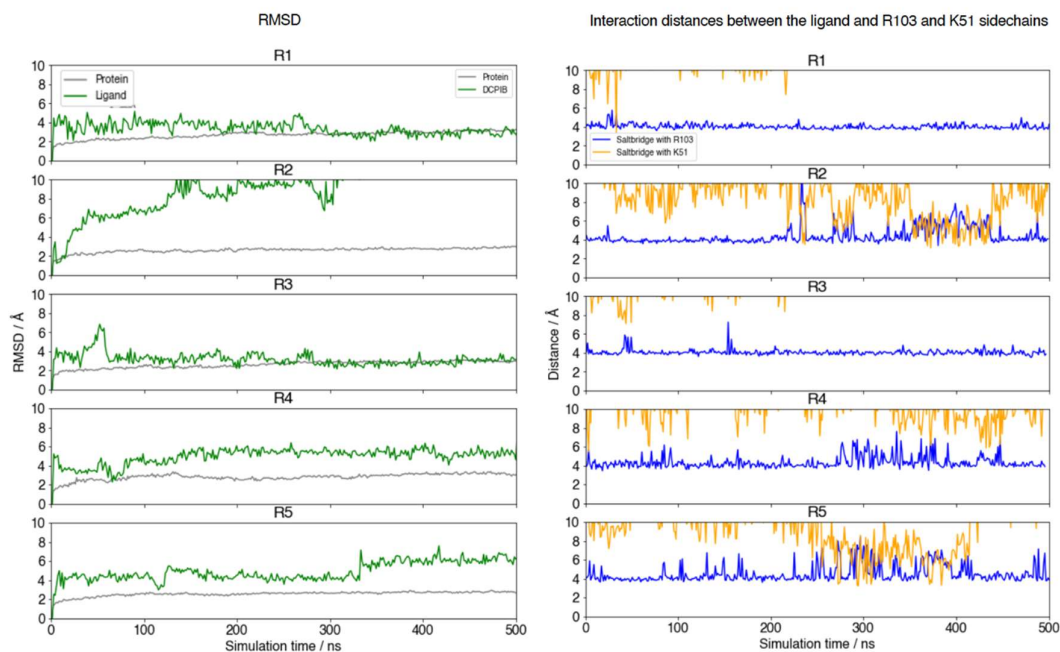


Figure S16. Structural analysis of the complex K51Q DCPIB - LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. **These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.**

DCPIB-Est - LRRRC8A:LRRRC8C (4:2) complex

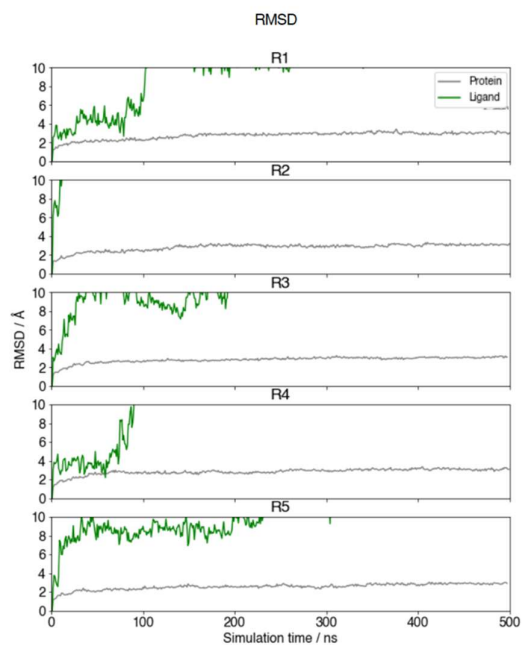


Figure S17. Structural analysis of the complex DCPIB-Est - LRRRC8A:LRRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. These data indicate stable equilibration of the system after the initial phase of the simulations.

1 - LRRC8A:LRRC8C (4:2) complex

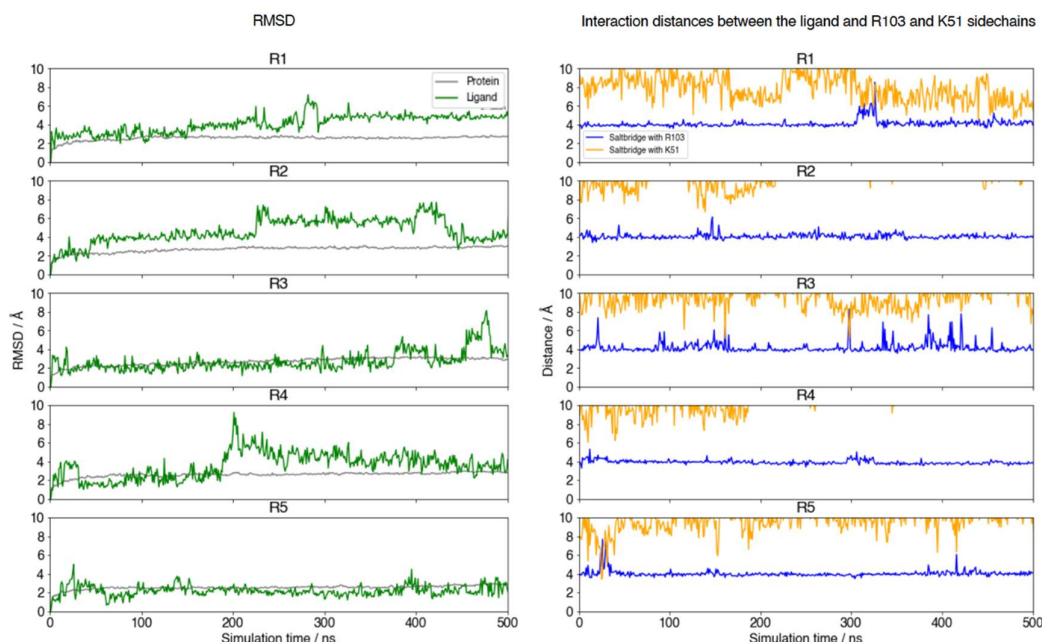


Figure S18. Structural analysis of the complex 1 - LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

1 - LRRC8A:LRRC8E (4:2) complex

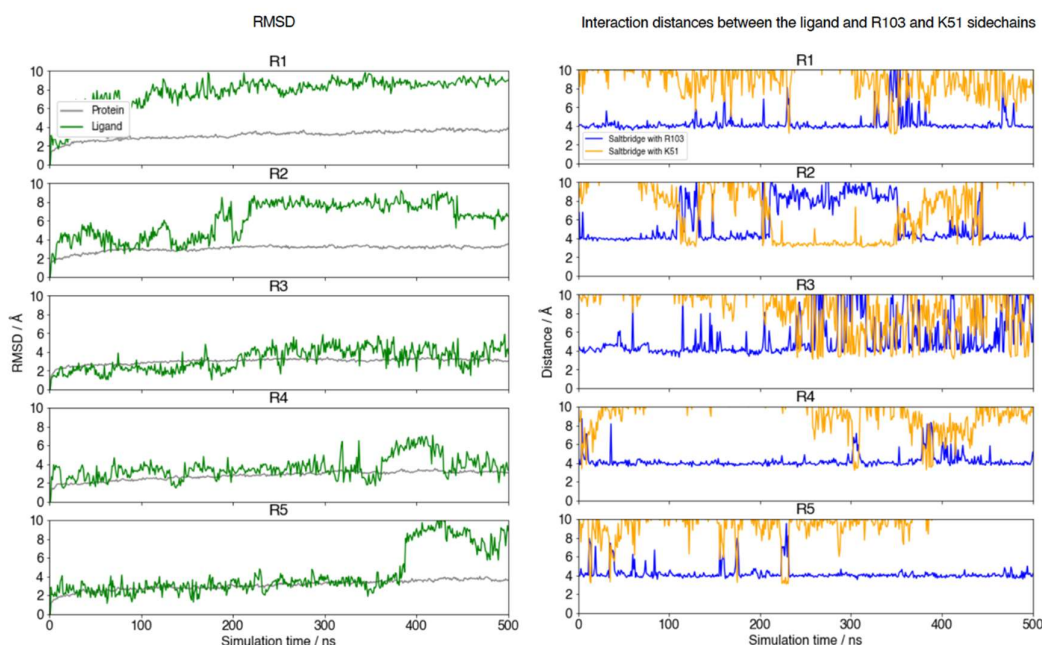
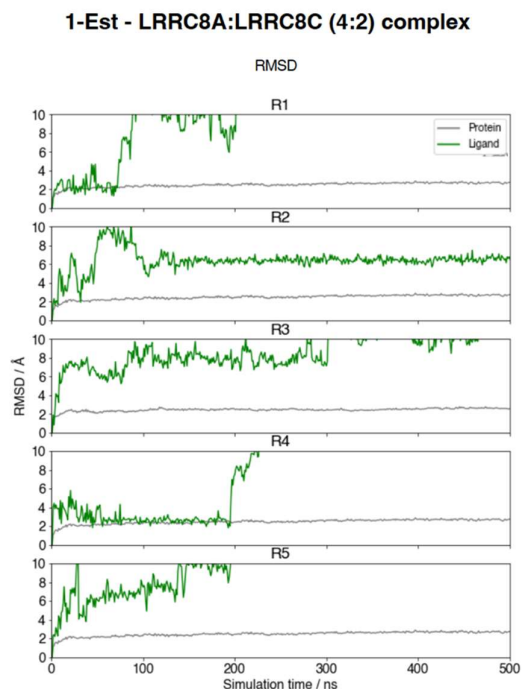


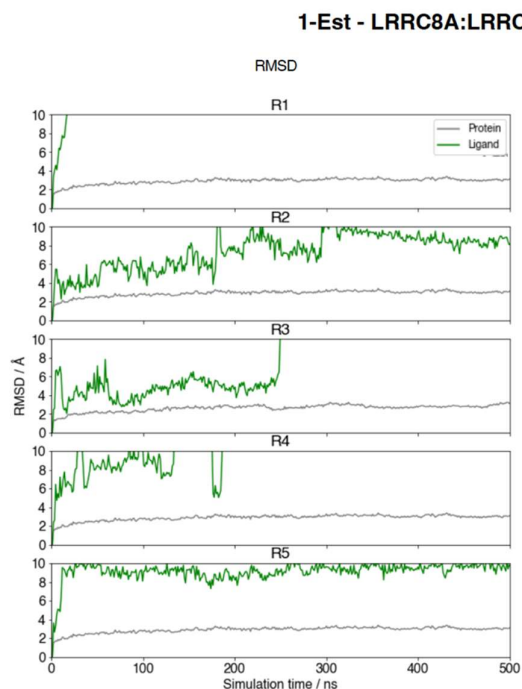
Figure S19. Structural analysis of the complex 1 - LRRC8A:LRRC8E (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

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Figure S20. Structural analysis of the complex 1-Est - LRRRC8A:LRRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. These data indicate stable equilibration of the system after the initial phase of the simulations.



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Figure S21. Structural analysis of the complex 1-Est - LRRRC8A:LRRRC8E (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. These data indicate stable equilibration of the system after the initial phase of the simulations.

2 - LRRC8A:LRRC8C (4:2) complex

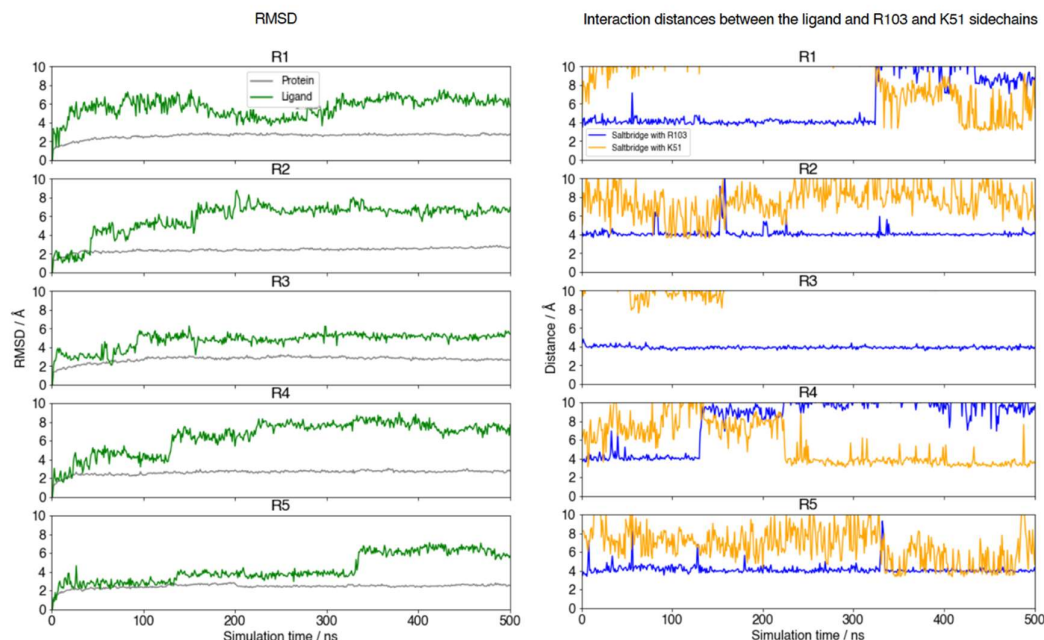


Figure S22. Structural analysis of the complex 2 - LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

2 - LRRC8A:LRRC8E (4:2) complex

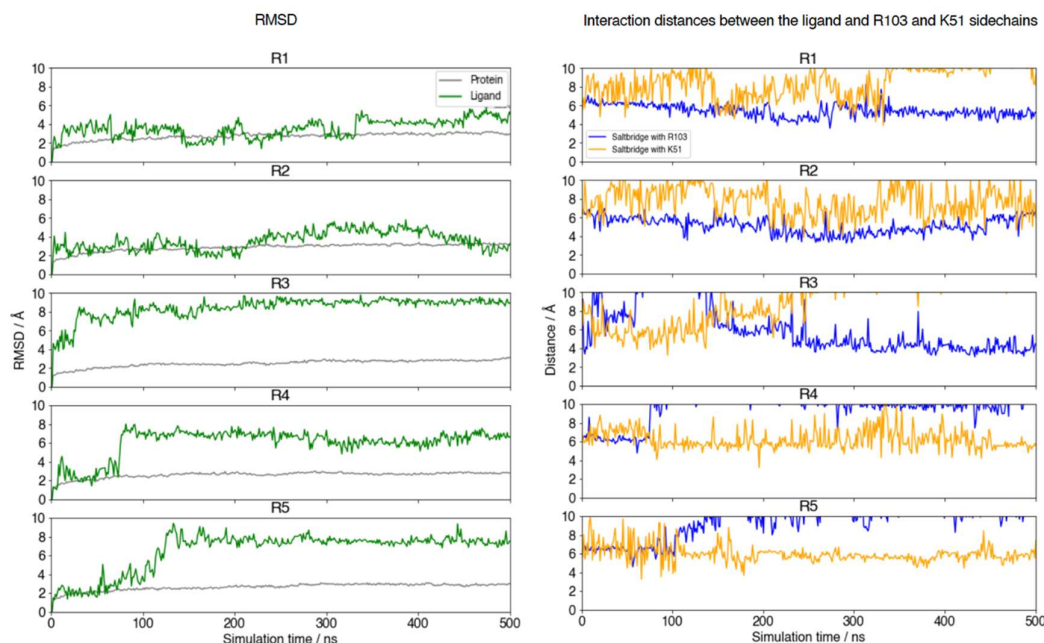
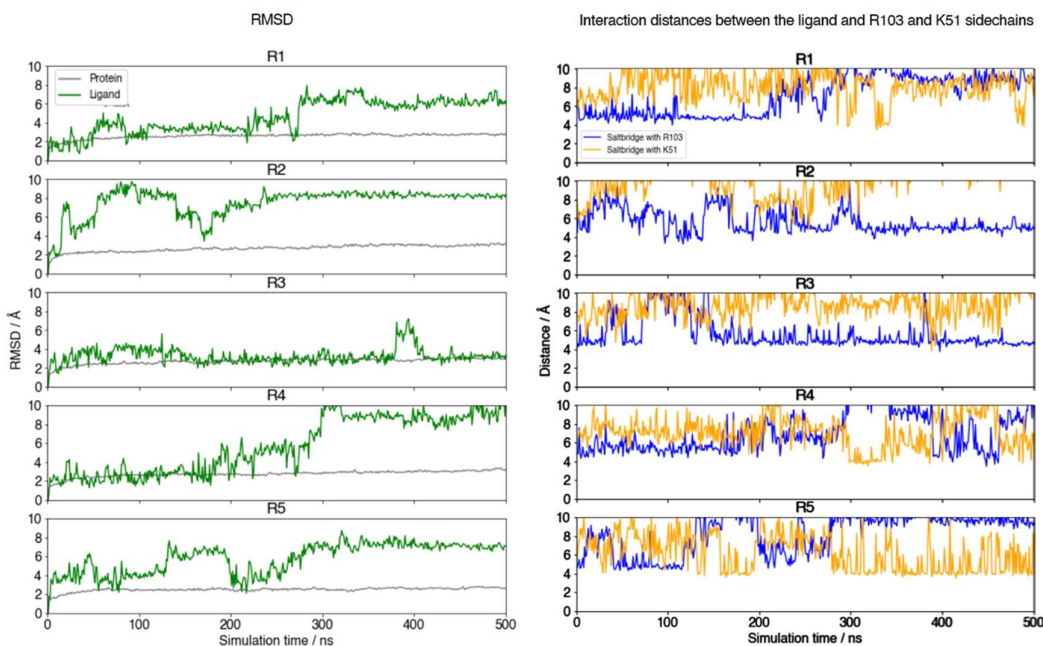


Figure S23. Structural analysis of the complex 2 - LRRC8A:LRRC8E (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

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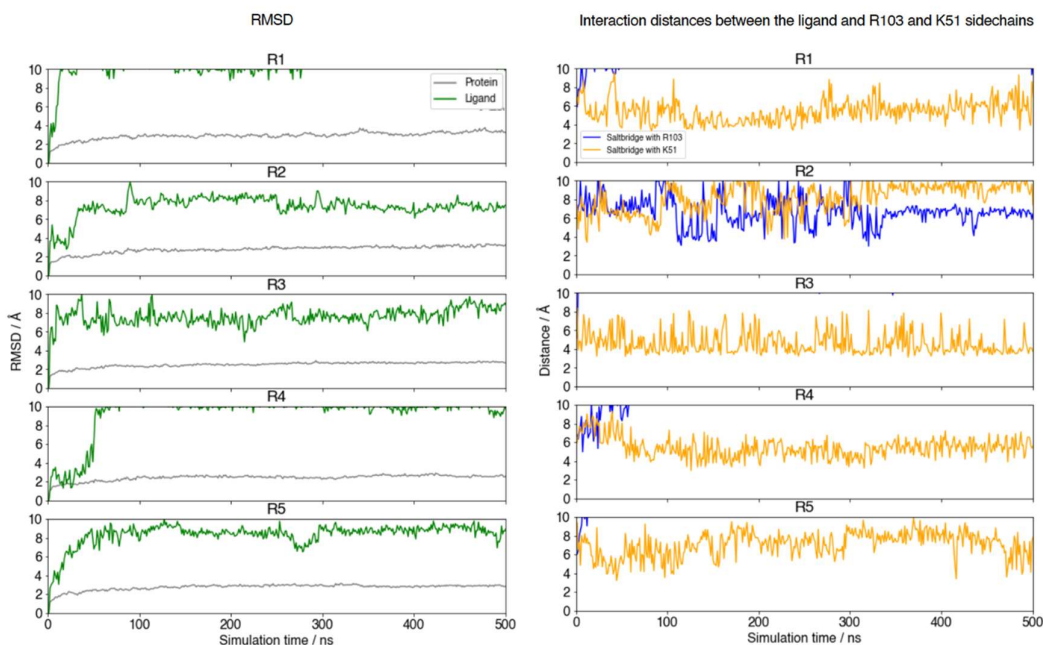
2-Est - LRRC8A:LRRC8C (4:2) complex



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Figure S24. Structural analysis of the complex 2-Est - LRRC8A:LRRC8C (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

2-Est - LRRC8A:LRRC8E (4:2) complex



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Figure S25. Structural analysis of the complex 2-Est - LRRC8A:LRRC8E (4:2) channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.

DCPIB - LRRC8A complex

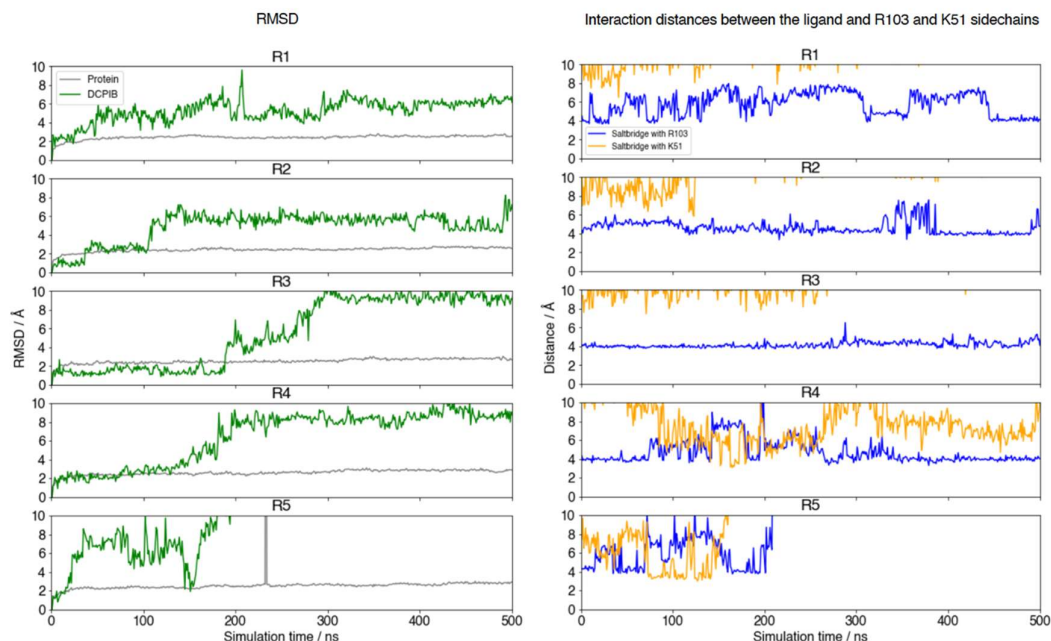


Figure S26. Structural analysis of the complex DCPIB - LRRC8A channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. **These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.**

DCPIB – R103F LRRC8A complex

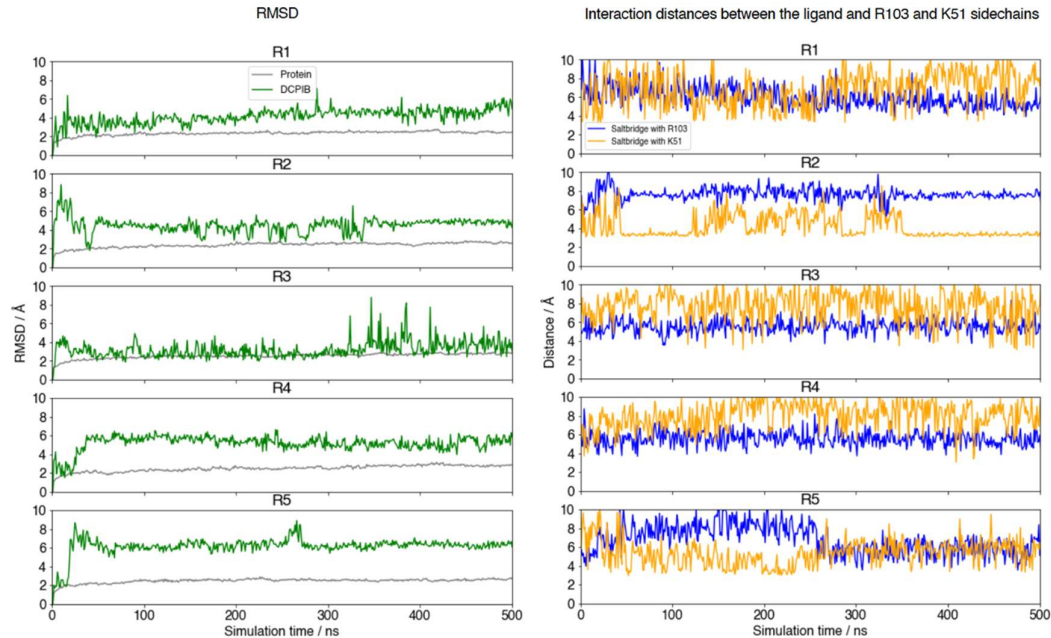


Figure S27. Structural analysis of the complex DCPIB – R103F LRRC8A channel. Root-mean-squared deviation (Å) along the simulations time of the system: heavy atoms of the ligand and the Ca atoms of the protein are shown in green and grey lines respectively. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the F103 sidechains and K51 sidechains (Å) along the simulations time shown as blue and orange lines respectively. **These distances report the persistence of interactions between the ligand and key residues in the selectivity filter.**

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****Section 3. Binding mode analysis and quantitative summary****

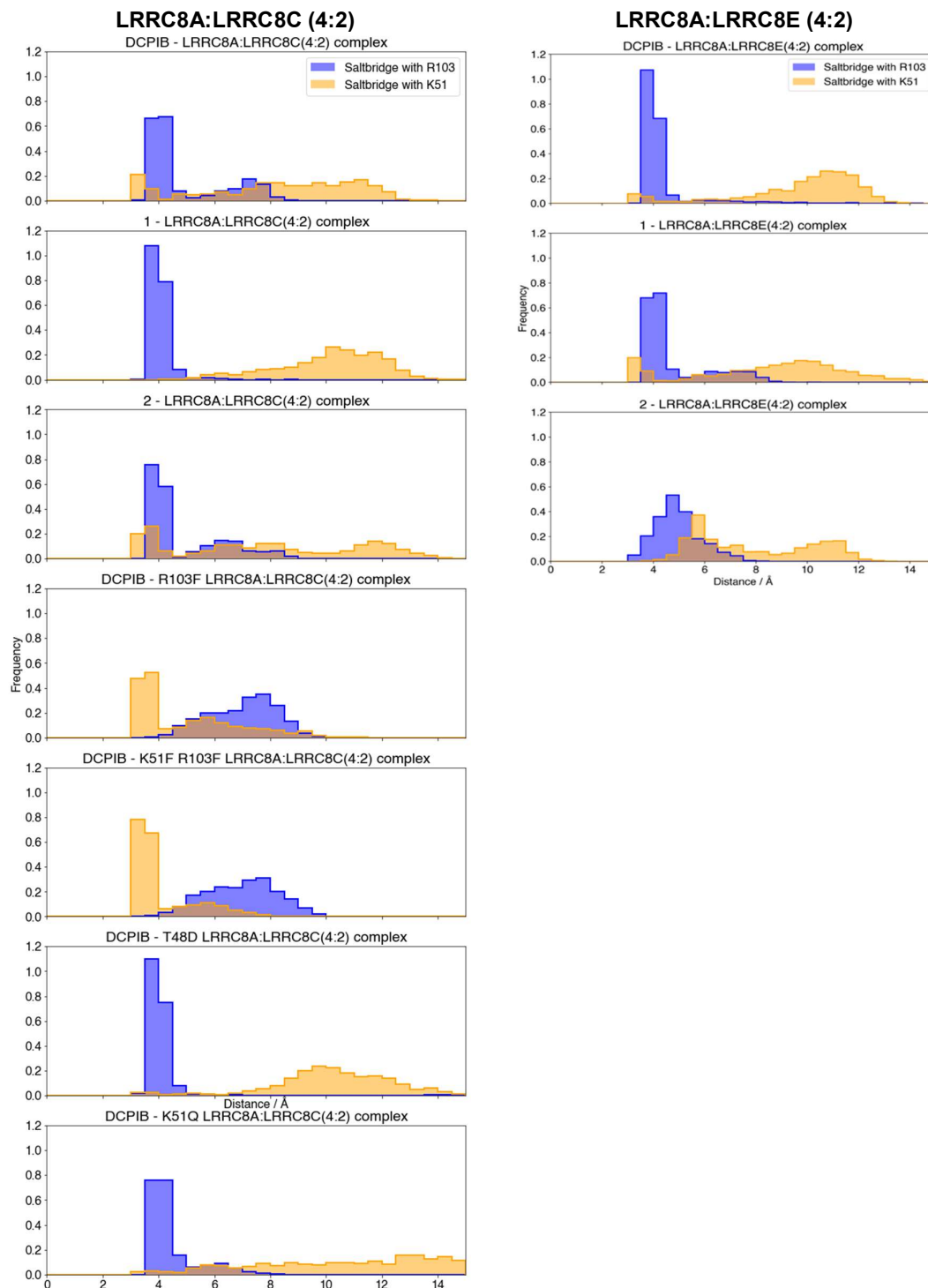
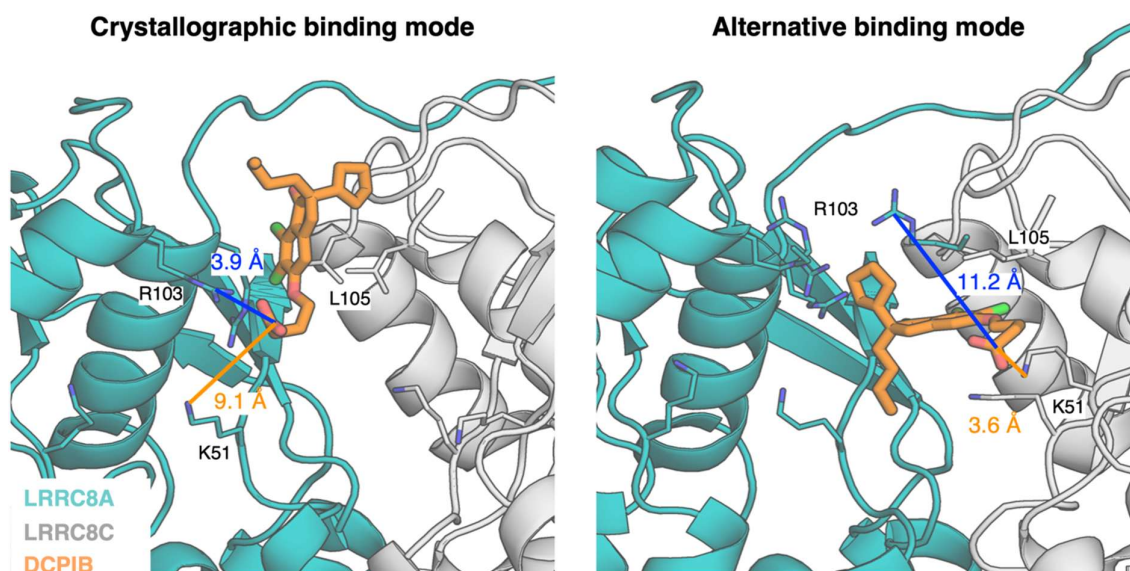


Figure S28. Histograms of the interaction distances between DCPIB and its analogues and the LRRC8A:LRRC8C (4:2) and LRRC8A:LRRC8E (4:2) channels. Histogram of the minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 / F103 sidechains and K51 / F51 / Q51 sidechains (Å) shown as blue and orange bars respectively. Histograms were calculated using the last 300ns of simulation time.

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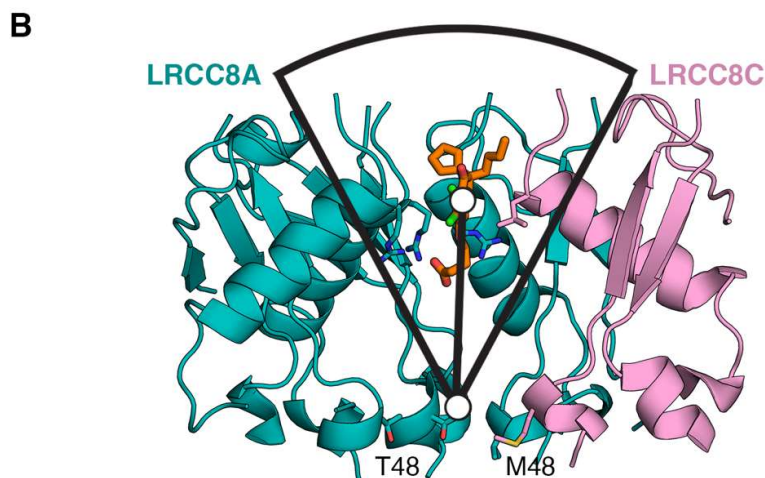


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Figure S29. Visualization of DCPIB binding modes and its interaction distances with the R103 and K51 sidechains. Minimum distances between the carboxylic acid of the ligand and the atom C_z of the R103 sidechains and K51 sidechains (Å) are shown as blue and orange lines respectively.

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hLRRC8A (Uniprot ID Q8IWT6):	43-	TLQVTQDKMICLPCK	-58
hLRRC8C (Uniprot ID Q8TDW0):	43-	TLQVMQDKIICLPCK	-58
hLRRC8E (Uniprot ID Q6NSJ5):	43-	TLQVTQDKIICLPNH	-58
hLRRC8A (Uniprot ID Q8IWT6):	100-	DLDRLQYNYVDAVCYENRLHWFAK	-123
hLRRC8C (Uniprot ID Q8TDW0):	100-	DLDLQQYSFINQMCYERLHWYAK	-123
hLRRC8E (Uniprot ID Q6NSJ5):	71-	NLDLQQYSFINQLCYETLHWYAK	-94
hLRRC8A (Uniprot ID Q8IWT6):	292-	DVDCTVDIESLTGYRTYRCAHPLATLF	-318
hLRRC8C (Uniprot ID Q8TDW0):	292-	DCNVDIQDMTGYNFSCNHTMAHLFSK	-318
hLRRC8E (Uniprot ID Q6NSJ5):	207-	LVACRVETSEVTGYASFCCNHTKAHLFSK	-235



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Figure S30. Computational details of the Steered molecular dynamics simulations. (A) Comparison of the sequences of the LRRC8A, LRRC8C and LRRC8E proteins included in the selectivity filter model. **(B)** Graphic description of the collective variable used in the Steered MD simulations. The collective variable is defined as the Euclidean distance between the center of mass of the 1-indanone scaffold and the center of mass the backbone of the residues at the T48 (LRRC8A), M48 (LRRC8C) and T48 (LRRC8E) and can take values from 18 to 33 Å.

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Table S1. Membrane composition of the transmembrane model used in the molecular dynamics simulations study described in this work.

	Outer leaflet	%	Inner leaflet	%
Cholesterol CHL	86	43	69	35
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine POPC	98	49	42	21
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-ethanolamine POPE	16	8	62	31
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphatidylserine POPS	0	0	25	13
Total	200		198	

Table S2. Summary table of the Molecular Dynamics simulations described in this work.

Model: Protein-membrane systems			
Simulated system		Ligand bound	Unbiased MD Simulations
LRRC8A:LRRC8C (4:2)	<i>wt</i>	-	5 replicates x 500ns
		ATP	5 replicates x 500ns
		DCPIB	5 replicates x 500ns
		DCPIB-Est	5 replicates x 500ns
		1	5 replicates x 500ns
		1-Est	5 replicates x 500ns
		2	5 replicates x 500ns
		2-Est	5 replicates x 500ns
	<i>R103F</i>	-	5 replicates x 500ns
		ATP	5 replicates x 500ns
		DCPIB	5 replicates x 500ns
	<i>K51F R103F</i>	-	5 replicates x 500ns
		DCPIB	5 replicates x 500ns
	<i>K51Q</i>	-	5 replicates x 500ns
		DCPIB	5 replicates x 500ns
LRRC8A:LRRC8E (4:2)	<i>wt</i>	-	5 replicates x 500ns
		ATP	5 replicates x 500ns
		DCPIB	5 replicates x 500ns
		DCPIB-Est	5 replicates x 500ns
		1	5 replicates x 500ns
		1-Est	5 replicates x 500ns
		2	5 replicates x 500ns
		2-Est	5 replicates x 500ns
LRRC8A	<i>wt</i>	DCPIB	5 replicates x 500ns
	<i>R103F</i>	DCPIB	5 replicates x 500ns

Model: reduced systems of the selectivity filter				
Simulated system		Ligand bound	unbiased MD Simulations	Steered MD simulations
LRRC8A:LRRC8E (4:2)	<i>wt</i>	DCPIB	4 replicas x 500ns	60 replicas x 30ns
		DCPIB-Est	4 replicas x 500ns	60 replicas x 30ns
		1	4 replicas x 500ns	60 replicas x 30ns
		1-Est	4 replicas x 500ns	60 replicas x 30ns
		2	4 replicas x 500ns	60 replicas x 30ns
		2-Est	4 replicas x 500ns	60 replicas x 30ns

Table S3. Table detailing percentages of each binding mode for each system described in this work. Binding modes were defined based on the interaction pattern between the ligand and residues R103 and K51 (or their corresponding mutants), as described in the main text. BM1 corresponds to the canonical binding orientation, whereas BM2 represents alternative interaction configurations.

Model: Protein-membrane systems									
Simulated system		Ligand bound		R1	R2	R3	R4	R5	Total
LRRC8A: LRRC8C (4:2)	wt	DCPIB	BM1	91%	84%	36%	76%	89%	75%
			BM2	6%	11%	41%	1%	1%	12%
		1	BM1	96%	99%	94%	99%	99%	98%
			BM2	2%	1%	0%	0%	1%	1%
		2	BM1	64%	93%	99%	25%	88%	77%
			BM2	12%	6%	0%	53%	9%	17%
	R103F	DCPIB	BM1	2%	3%	1%	28%	8%	9%
	BM2		65%	80%	66%	5%	53%	52%	
	K51F R103F	DCPIB	BM1	1%	1%	9%	10%	29%	12%
	BM2		52%	51%	60%	44%	32%	33%	
K51Q	DCPIB	BM1	99%	81%	98%	88%	80%	89%	
BM2		0%	8%	0%	2%	4%	2%		
T48D	DCPIB	BM1	92%	99%	91%	33%	95%	83%	
BM2		8%	0%	0%	1%	1%	2%		
LRRC8A: LRRC8E (4:2)	wt	DCPIB	BM1	100%	70%	95%	95%	89%	85%
			BM2	0%	22%	4%	1%	1%	10%
		1	BM1	92%	63%	68%	93%	94%	82%
			BM2	2%	32%	12%	3%	2%	10%
		2	BM1	74%	77%	63%	2%	4%	44%
			BM2	9%	16%	17%	56%	61%	31%