Supplementary Material

Ionic Coulomb Blockade and the Determinants of Selectivity in the NaChBac Bacterial Sodium Channel

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S1. Electrophysiology

Table S1. Oligonucleotides containing the sequence for the desired amino acid substitutions used in this study.

| Amino acid sequence | Nominal Qf | Template | Forward primer | Reversed primer |
|---------------------|------------|----------|----------------|-----------------|
| LASWAS              | 0          | LESWAS   | GGTACGCTAGcctCATGGCGAGGggcg  | ACTTGGAACAATGTTAACAACGtaagc |
| LEKWAS              | 0          | LESWAS   | CACGCTAGAGaagTGGCGGAGCG     | ACCACTTGGAACAATGTTAAC |
| LDSWAS              | -4         | LESWAS   | GGTACGCTAgatTACGGCGAGG     | ACTTGGAACAATGTTAACAACGtaagc |
| LEEWAS              | -8         | LESWAS   | CACGCTAGAGGagTGGCGGAGCG    | ACCACTTGGAACAATGTTAAC |
| LEDWAS              | -8         | LESWAS   | CACGCTAGAGGagTGGCGGAGCG    | ACCACTTGGAACAATGTTAAC |
| LDEWAS              | -8         | LEEWAS   | GGTACGCTAgatGAGTGGCGGA     | ACTTGGAACAATGTTAACAACGtaagc |
| LDDWAS              | -8         | LEDWAS   | GGTACGCTAgatGATTGGCGGA     | ACTTGGAACAATGTTAACAACGtaagc |

Whole-cell voltage clamp recordings were performed at room temperature (20°C) using an Axopatch 200A (Molecular Devices, Inc.) amplifier. Whole cell currents were elicited by a series of step depolarizations (+95mV to -85mV in -15mV steps) from $V_{\text{hold}} = -100mV$. Representative examples of currents recorded under such conditions, mediated by wild type and mutant NaChBac channels, are shown on Figure S0.

Whole-cell rather than single-channel recordings are used in the interests of precision, based on the larger and more easily measurable currents. The amplitude of a single NaChBac channel is only about 2 pA in 140 mM Na solution, so if the permeability of a channel decreases two-fold or even more in solutions with other tested cations, it would be difficult to detect the currents and obtain reliable values for conductances. The whole cell peak currents of wild type NaChBac in 140 mM Na solution have amplitudes of 1-2 nA, which enables us to perform reliable recordings. Whole-cell currents are the product of the number of channels, the open probability and the single channel conductance. Because the number of expressed channels is constant for a given cell, and the open probability does not change with a change of the tested cation in solution, the difference in conductance observed is proportional to the change in single channel conductance.

Patch-clamp pipettes were pulled from borosilicate glass (Kimax, Kimble Company, USA) to resistances between 2-3 MOhm. Pipette solution was either PS1 (120mM Cs-methanesulfonate, 20mM Na-gluconate, 5mM CsCl, 10mM EGTA, and 20mM HEPES, pH7.4 adjusted with 1.8mM
CsOH) or Cs-free PS2 (15mM Na-gluconate, 5mM NaCl, 90mM NMDG, 10mM EGTA, and 20mM HEPES, pH7.4 adjusted with 3mM HCl). Unless otherwise stated, GOhm seals were obtained in standard bath solution (SBSNa: 140mM Na-methanesulfonate, 5mM CsCl, 10mM HEPES and 10mM glucose, pH=7.4 adjusted with 4.8mM CsOH). For experiments with LDDWAS and LDEWAS, Cs-free SBSNa (SBSNa: 132mM Na-methanesulfonate, 5mM NaCl, 10mM HEPES and 10mM glucose, pH=7.4 adjusted with 3.6mM NaOH) was used; these channels are permeable to Cs and thus its removal from the test solutions was required to establish pseudo-bionic conditions (Fig. S1). Furthermore, the bath solutions (SBSNa and SBSNa in which 140 mM Na⁺ was replaced with the test monovalent cation) used for recording LDDWAS activity in the presence of extracellular monovalent cations also contained 10 mM EGTA (see below) to account for the high affinity Ca2+ blockade of the channel.

Permeability to different test cations was determined by replacing 140mM NaCl with equimolar test monovalent cation or 100mM test divalent cation using Cl⁻ salts. Permeability ratios (Pₓ/PNa) were determined from whole cell current reversal potentials (E_{rev}) for monovalent/divalent cations according to [34, 35]. The effective activity coefficients were calculated using the Debye-Hückel equation (Table S2).

In experiments with varied Na/Ca mixtures, GOhm seals were first obtained in SBSNa (or SBSNa for experiments with LDDWAS and LDEWAS) containing 10 nM [Ca^{2+}]_{free}, and the bath solution replaced with solutions containing increasing concentrations of Ca^{2+}. Different chelators were used to fix Ca²⁺ concentration as needed, for details see Table S3., and HEDTA to fix Ca²⁺ concentrations at 10 µM, 100 µM and 1 mM. [Ca^{2+}]_{free} were calculated using Webmaxc (http://web.stanford.edu/cpatton/webmaxcs.html). Na⁺ and Ca²⁺ (for 10mM, 50mM and 100mM) activities were calculated using the Debye-Hückel equation (we consider [Ca^{2+}]_{free} = ion activity of Ca^{2+}); for details see Table S2.

Osmolarities of all solutions were measured using a Wescor vapor pressure osmometer (model 5520) and adjusted to 280 mOsm/kg⁻¹ using sorbitol. All solutions were filtered with a 0.22mm filter before use. Whole cell currents were recorded 3 minutes after obtaining whole cell configuration to ensure complete equilibration of the pipette solution and cytosol. The bath solution was grounded using a 3 M KCl agar bridge; the liquid junction potential, determined experimentally [36], agreed with that calculated (using JPCalc program, Clampex, Axon Instruments, Inc.), were less than 2.6mV, and were not accounted for.

The recording chamber volume was approximately 200 µl and was continuously exchanged by a gravity-driven flow/suction arrangement at rate of ≈2ml/min; to ensure complete exchange of bath solution. Electrophysiological recordings were initiated after >4 minutes of continuous solution change.

Results were analysed using Clampfit 10.1 software (Molecular Devices) and Origin 9.1 (OriginLab). Data are presented as Mean (± SEM) (n), where n is the number of independent experiments.
Table S2. Concentrations and the effective activity coefficients of solutions used for permeability ratios determination.

|                      | Na$^+$ | Li$^+$ | K$^+$ | Rb$^+$ | Cs$^+$ | Mg$^{2+}$ | Ca$^{2+}$ | Sr$^{2+}$ | Ba$^{2+}$ |
|----------------------|--------|--------|-------|--------|--------|-----------|-----------|-----------|-----------|
| Concentration (in mM)| 140    | 140    | 140   | 140    | 140    | 100       | 100       | 100       | 100       |
| activity coefficients (Debye-Hückel equation) | 0.74   | 0.78   | 0.72  | 0.71   | 0.71   | 0.34      | 0.29      | 0.25      | 0.25      |
| Free concentrations/activities | 103.6  | 109.2  | 100.8 | 99.4   | 99.4   | 34        | 29        | 25        | 25        |
**Table S3.** Total and free concentrations, which correspond to ion activities, for the mixed Na\(^+\) and Ca\(^{2+}\) solutions in our AMFE experiments (*only for LDDWAS and LDEWAS mutant channels).

| Solutions (total concentrations) | Free concentrations/ ion activities |
|----------------------------------|------------------------------------|
| Na\(^+\) | Ca\(^{2+}\) | Chelator                        | Na\(^+\) | Ca\(^{2+}\) |
| 140 mM | 0.23 mM | 12 mM EGTA + 10 mM BAPTA*       | 110.6 mM | 0.12 nM*   |
| 140 mM | 0.23 mM | 12 mM EGTA                      | 110.6 mM | 1.5 nM     |
| 140 mM | 0.23 mM | 3 mM EGTA                       | 110.6 mM | 6.4 nM     |
| 140 mM | 0.23 mM | 2 mM EGTA                       | 110.6 mM | 10 nM      |
| 140 mM | 0.85 mM | 2 mM EGTA                       | 110.6 mM | 100 nM     |
| 140 mM | 1.76 mM | 2 mM EGTA                       | 110.6 mM | 1 µM       |
| 140 mM | 0.69 mM | 1 mM HEDTA                      | 110.6 mM | 10 µM      |
| 140 mM | 1.05 mM | 1 mM HEDTA                      | 110.6 mM | 100 µM     |
| 138 mM | 2 mM    | 1 mM HEDTA                      | 108.7 mM | 1 mM       |
| 130 mM | 10 mM   |                                  | 101.0 mM | 4.13 mM    |
| Concentration 1 | Concentration 2 | Concentration 3 | Concentration 4 |
|----------------|----------------|----------------|----------------|
| 70 mM          | 50 mM          | 52.2 mM        | 18.3 mM        |
| 40 mM          | 100 mM         | 28.4 mM        | 31.7 mM        |
Figure S0. Examples of raw experimental measurements of monovalent (A, B) and divalent (C, D) ionic currents mediated by wild type LESWAS (A, C) and mutant LEDWAS (B, D) NaChBac channels as functions of time following step depolarisations. The sodium currents, used for normalisation purposes, are shown in panel E.
S2. Experimental results of mutant studies of NaChBac

Table S4. Reversal potentials (\(E_{\text{rev}}\)), permeability ratios (\(P_X/P_{Na}\)) and the relative peak inward current (\(I_X/INa\)) for the tested cations for the channels with \(Q_f = -4\) and \(Q_f = -8\). All values are means (± SEM) with the number of experiments in parenthesis. \(E_{\text{rev}}\) is the mean reversal potential in millivolts measured for each cation; in cases where inward current was not detected, estimated values for \(E_{\text{rev}}\) were determined as voltage at which outward current could be detected. Permeability ratios (\(P_X/P_{Na}\)) for each cation were calculated (see in Methods) from paired changes in \(E_{\text{rev}}\) measured for a given cell perfused first with control Na\(^+\) solution and after replacement with the test cation solution according to the following equations (Hille, 1972; Sun et al., 1997) for monovalent cations:

\[
P_X/P_{Na} = \frac{\alpha Na_i}{\alpha X_e} \left[ \exp \left( \frac{\Delta E_{\text{rev}} (RT)}{F} \right) \right],
\]

and divalent cations:

\[
P_Y/P_{Na} = \frac{\alpha Na_i [\exp (E_{\text{rev}} F/RT)][\exp (E_{\text{rev}} F/RT)+1]}{4\alpha Y_e},
\]

where \(\Delta E_{\text{rev}}\) is the change in reversal potential on replacing Na\(^+\) with the tested cation, \(\alpha\) is the activity coefficients for the ion (i, internal and e, external), R - the universal gas constant, T - absolute temperature, and F - the Faraday constant. The effective activity coefficients (\(\alpha x\)) were calculated using the Debye-Hückel equation and are listed in Supplemental Table 2. \(I_X/INa\) was measured as the ratio of maximum peak inward current observed for the test cation to that for observed Na\(^+\) in the same cell.

|       | LESWAS     | LDSWAS     | LEEWAS     | LEDWAS     | LEDWAS     | LDDWAS     |
|-------|------------|------------|------------|------------|------------|------------|
| Li\(^+\) E_{\text{rev}} | 48.7±1.42 mV (n=6) | 42.3±1.8 mV (n=6) | 47.9±0.4 mV (n=9) | 43.3±2.1 mV (n=6) | 48.2±1.57 mV (n=6) | 42.3±0.7 mV (n=7) |
|       | P_{Li}/P_{Na} | 0.8       | 0.6       | 0.7       | 0.6       | 0.8       | 0.6       |
|       | I_{Li}/I_{Na} | 0.8       | 0.6       | 0.7       | 0.9       | 0.7       | 1         |
| Na\(^+\) E_{\text{rev}} | 51.0±1.26 mV (n=17) | 49.0±1.0 mV (n=21) | 51.8±1.1 mV (n=16) | 47.8±1.4 mV (n=10) | 50.2±0.81 mV (n=14) | 46.4±1.0 mV (n=14) |
|       | P_{Na}/P_{Na} | 1         | 1         | 1         | 1         | 1         | 0.9       |
|       | $I/I_{Na}$ | 1  | 1  | 1  | 1  | 1  | 1  |
|-------|------------|----|----|----|----|----|----|
| $K^+$ | $E_{rev}$  | <-30 mV | 42.5±2.4 mV | -15.9±3.8 mV | 43.4±3.1 mV | 9.7±2.31 mV | 48.5±0.6 mV |
|       |            | (n=8) | (n=5) | (n=7) | (n=7) | (n=8) | (n=7) |
| $Rb^+$| $E_{rev}$  | <-30 mV | -8.8±6.0 mV | -22.9±4.7 mV | 10.3±1.5 mV | -7.9±1.57 mV | 35.9±4.1 mV |
|       |            | (n=5) | (n=5) | (n=6) | (n=5) | (n=6) | (n=7) |
| $Cs^+$| $E_{rev}$  | <-30 mV | -29.0±5.0 mV | -40.4±6.7 mV | -3.9±3.1 mV | -11.8±7.42 mV | 8.2±1.1 mV |
|       |            | (n=5) | (n=5) | (n=6) | (n=5) | (n=6) | (n=7) |
| $Mg^{2+}$| $E_{rev}$ | -26.3±9.24mV | 19.0±3.0 mV | -3.9±2.2 mV | 15.9±7.4 mV | 49.5±1.78 mV | Block |
|       |            | (n=5) | (n=6) | (n=6) | (n=5) | (n=6) | (n=15) |
| $Ca^{2+}$| $E_{rev}$| -5.1±2.63 mV | 53.3±1.2 mV | 58.7±1.8 mV | block | 71.5±1.26 mV | Block |
|       |            | (n=9) | (n=7) | (n=9) | (n=11) | (n=16) | |
| $Sr^{2+}$| $E_{rev}$| <-30 mV | 54.8±2.1 mV | 45.0±3.8 mV | 75.8±1.8 mV | 59.2±4.34 mV | Block |
|       |            | (n=5) | (n=6) | (n=7) | (n=7) | (n=15) | |

$P_{X/P Na}$

|       | 0.8 | <0.1 | 0.8 | 0.2 | 1  |
|-------|-----|------|-----|-----|----|
|       | 0.6 | <0.1 | 0.8 | 0.1 | 1.2|
|       | 0.1 | 0.1  | <0.1 | 0.2 | 0.6|
|       | <0.1 | <0.1 | <0.1 | 0.1 | <0.1 | 0.2|
| $P_{X/P Na}$ | 0.1 | 0.7 | 0.2 | 0.6 | 6.5 | - |
|       | <0.1 | <0.1 | <0.1 | 0.1 | <0.1 | <0.1 | 0.1 |
| $P_{X/P Na}$ | 0.1 | 0.4 | 0.25 | 0.4 | - |
|       | <0.1 | 0.4 | 0.25 | 0.4 | - |

**Block**
|      | $P_{Sr}/P_{Na}$ | $I/I_{Na}$ | $I/I_{Na}$ | $E_{rev}$ | $P_{Ba}/P_{Na}$ | $I/I_{Na}$ |
|------|----------------|------------|------------|----------|----------------|------------|
|     | $<0.1$         | 13.2       | 8.5        | 66.8     | 18.5           | -          |
|     |                | 0.4        | 0.2        | 0.3      | 0.3            | -          |
| $Ba^{2+}$ | $<-30$ mV     | $40.9\pm3.8$ mV | $13.4\pm0.8$ mV | $61.6\pm2.5$ mV | $49.0\pm1.98$ mV | Block |
|     | (n=5)          | (n=6)      | (n=8)      | (n=5)    | (n=9)          | (n=15)     |
|     | 4.7            | 0.7        | 22.2       | 8.5      |                | -          |
|     | $<0.1$         | 0.2        | $<0.1$     | 0.1      | 0.1            | -          |
Figure S1. Optimising the pipette solution. Plot of average peak current ($I_{\text{peak}}$) density against test voltage from cells expressing LDSWAS (A; ▲ - n = 10; ○ - n = 15; note the scale); LEEWAS (B; ▲ - n = 16; ○ - n = 7), LDDWAS (C; ▲ - n = 7; ○ - n = 14) and LDEWAS (D; ▲ - n = 10; ○ - n = 10) mutant channels recorded in SBS_Na and PS1 (▲) or PS2 (○) in response to test voltages ranging from +95 mV to -70 mV (in -15 mV steps) from $V_{\text{hold}}$ -100 mV. Note that for LDDWAS (and, to a lesser extent, LDEWAS) the removal of Cs$^+$ (○) from the pipette solution resulted in a shift in $E_{\text{rev}}$ and larger inward current consistent with Cs$^+$ permeation.

Figure S2. Original traces for LASWAS (A, B) and LEKWAS (C, D) mutant channels recorded in the bath solution (SBSNa) containing 140 mM NaCl (A, C) or in 100 mM CaCl$_2$ solution (B, D) and PS1 in response to test voltages ranging from +95 mV to -70 mV (in -15 mV steps) from $V_{\text{hold}}$ -100 mV.
Figure S3. Monovalent cation permeability. Mean peak current voltage relationships for wild type NaChBac LESWAS (a), LDSWAS (b), LEEWAS (c), DEWAS (d), LEDWAS (e) and LDDWAS (f) mutant channels for Na\(^+\), Li\(^+\), K\(^+\), Rb\(^+\) and Cs\(^+\) (as labelled) were determined by normalising peak current magnitudes recorded in Na\(^+\) bath solution from the same cell prior to replacement of extracellular Na\(^+\) for test cation. Averages (±SEM) are from at least 5 cells.
Figure S4. Divalent cation permeability. Mean peak current voltage relationships for wild type NaChBac LESWAS (a), LDSWAS (b), LEEWAS (c), LDEWAS (d), LEDWAS (e) and LDDWAS (f) mutant channels for Na⁺ (for comparison), Mg²⁺, Ca²⁺, Sr²⁺ and Ba²⁺ (as labelled) were determined by normalising peak current magnitudes recorded in Na⁺ bath solution from the same cell prior to replacement of extracellular Na⁺ for test cation. Averages (±SEM) are from at least 5 cells.
S3. Models and theories

Despite their small size, ion channels are complicated objects made up of thousands of atoms each of which interacts with all of the others, with water, and with any ions that are within the channel's pore or nearby. In practice, before physics can usefully be applied, some simplification is needed, and there are several different levels on which channels can be modelled. The features and mechanisms of the electro-diffusive ion motion have been the subject of numerous theoretical and simulation-based studies, performed with very different scales, models and methods [1, 2], including (in order of decreasing model detail) all-atom molecular dynamics (MD) simulations [3], mesoscopic Brownian dynamics (BD) simulations [2, 4], Monte-Carlo simulations [5], and Poisson-Nernst-Planck (PNP) simulations [6]. The different models represent different physical scales and provide complementary information. Here, we are mainly interested in the BD level, but with some illumination from MD modelling. To set the context, we now consider briefly the advantages and disadvantages of the latter two approaches.

Molecular dynamics modelling ignores electronic degrees of freedom but, in principle, considers all interactions contributing to the net force on each component particle and takes explicit account of individual water molecules. The atoms are usually treated as point particles, but with inclusion of hard-core repulsive forces to model the atomic radii. Sometimes, particular groups of atoms far from the pore are assumed to move together as single units in order to simplify and accelerate the computation. The main advantages of MD modelling lie in its conceptual simplicity and its inclusivity of all the different interactions, and it is often considered as the de facto standard for nanoscale research, quite generally, as well as for research on ion channels. It can be used to confirm the atomic structure of the channel protein, as well as to model the permeation process. Its main disadvantage is that the detailed character of the computations makes them very demanding in terms of computational resources so that, even using supercomputers and massive parallelism, it is currently seldom impossible to run the simulations for long enough to produce statistically meaningful currents for comparison with experiment. An important consequence is that MD cannot identify emergent phenomena at a higher level, for example ionic Coulomb blockade (ICB).

Unlike MD simulations [2], the BD model takes no account of the detailed atomic structure of the protein or residues. Rather, it treats the water and protein as continuum dielectrics with their bulk dielectric constants; unlike PNP theory, however, it takes explicit account of the charge/entity discreteness of the ions. Simplified electrostatically-controlled self-consistent BD models of this kind have already shown their utility for describing relatively wide calcium/sodium channels [4, 7, 15, 16]. They can be applied to e.g. TRP channels [14], to biomimetic nanotubes [11, 12], and to other artificial pores. The model is summarised below in section S3.1. Further details together with a fuller discussion of its validity and limitations, have been given elsewhere [9, 13, 14]. Its great advantage lies in computational speed, so that it is feasible not only to determine the permeating current with good statistics, but to do so under a wide range of different conditions, e.g. for different membrane potentials and ionic concentrations, as well as for different mutants.

As in the main paper, with SI units, $\varepsilon_0$ is the permittivity of free space, $e$ is the elementary charge, $z$ is the ion valence, $k_B$ is Boltzmann’s constant and $T$ is the temperature. We use the conventional shorthand symbols for amino acid residues: Alanine (A); Aspartate (D, with $Q = -1|e|$); Glutamate (E, with $Q = -1|e|$); Leucine (L); Lysine (K, with $Q = +1|e|$); Serine (S); Threonine (T); Tryptophan (W); and so on, where A, L, S, T, and W are all uncharged.

S3.1. Self-consistent electrostatic & Brownian dynamics model

The generic electrostatic/BD model describes the SFs of calcium/sodium ion channels. It treats the channel’s SF as a water-filled, cylindrical, negatively-charged pore in the protein, radius $R_c \approx 0.3\text{nm}$ and length $L \approx 1\text{nm}$. Such simple models have been widely and successfully used in earlier research [4, 7, 15, 16] to describe the permeation of small metallic cations. The model is shown schematically in Fig. S3(a). The $x$-axis is coincident with the channel axis and $x = 0$ in the center of channel. The charged residues are modelled as a symmetrically-placed, uniformly-charged, rigid ring $R_Q \leq R_c$ of negative charge $|Q_f| = (0 - 8)e$. We
Figure S 5: Electrostatic model of the selectivity filter (SF) of a \( \text{Ca}^{2+} \) or \( \text{Na}^+ \) channel. (a) The model represents the SF as a negatively-charged, axisymmetric, water-filled, cylindrical pore of radius \( R_c \approx 0.3 \text{nm} \) and length \( L_c \approx 1.6 \text{nm} \) through the protein hub in the cellular membrane. The protein is pale-blue-shaded and the water is colourless. The fixed charge \( Q_f \) is modelled as a single charged ring of radius \( R_Q \), shaded in red; in the present manuscript we take no account of the possible difference in radii of the channel and the charge ring, but assume that \( R_Q = R_c \). (b) Energetics of a moving \( \text{Ca}^{2+} \) ion for a fixed charge \( Q_f = -1e \). The dielectric self-energy barrier \( U_{SE}^{Q_f} \) (dashed blue line) is balanced by the site attraction \( U_{Q_f} \) (dashed green line) resulting in an almost barrier-less energy profile \( U_b \) (red solid line). (Modified from \[24\])

take both the water and the protein to be homogeneous continua with dielectric constants \( \varepsilon_w = 80 \) and \( \varepsilon_p = 2 \), respectively. Despite its simplicity, the model allows one to predict/explain some essential features of conduction and selectivity and, in particular, valence selectivity \[17\] including ICB effects \[7, 14, 18\]; as mentioned above, the model was recently extended to account for local binding \[19\].

Fig. S5(b) illustrates the phenomenon of resonant barrier-less conduction, which is typical of electrostatic models. It arises when the energy of the ion-site attraction \( U_{Q_f} \) balances the dielectric self-energy (or more generally, the dehydration) barrier \( U_{SE}^{Q_f} \). Resonant barrier-less conduction is key to the understanding of selectivity in ion channels \[9, 14, 20, 21\]. Selectivity arises because different ion species exhibit different barrier heights and site affinities, and have different dependences of these parameters on \( Q_f \) and ionic radii.

The model supposes that ion transport through the SF is controlled by coupled Poisson electrostatic and Langevin stochastic equations \[2, 4\] i.e. that we can implement self-consistent Brownian dynamics (BD) simulations of ion transport. Single-file ionic motion is assumed for \( \text{Ca}^{2+} \) and \( \text{Na}^+ \) channels. The BD simulations are based on numerical solution of the 1D over-damped, time-discretised, Langevin equation for the \( i \)-th ion. A parametric study \[15\] showed that the results are robust to small variations in the geometrical parameters of the SF.

As in the case of other simplified models, we make use of effective values of many model parameters, such as \( R, L, \varepsilon_w, \varepsilon_p \) and, particularly \( Q_f \). The concept of effective values allows one to use very generalised models far outside the range of rigorous validity, e.g. for more complicated geometry of the SF. The effective charge \( Q_f^* \) was introduced as a fitting parameter to optimise agreement of the model with theoretical or experimental data. The physical nature of \( Q_f^* \) depends on the particular model used, and on the channel structure, and \( Q_f^* \) may differ from \( Q_f^{nm} \) on account of e.g. the dipole moment of the molecule, screening, or site protonation.

We will use the effective charge \( Q_f^* \) as opposed to the nominal charge \( Q_f^{nm} \) and, as discussed in detail below, we will hypothesise that the difference between them is due to protonation (see Sec. S3.5).
S3.2. Ionic Coulomb blockade

For completeness, and for convenience of the reader, we now provide a brief description of the ICB model of permeation and selectivity in biological ion channels. We follow fairly closely the Wikipedia article “Ionic Coulomb blockade”.

ICB is a fundamental electrostatic phenomenon that emerges in the electro-diffusive transport of ions through narrow, low-capacitance channels, whether biological or artificial. ICB predicts resonant (or zero-conduction) points to be an important determinant of selectivity, and one that is manifested strongly for divalent ions e.g. by giving rise to Ca\(^{2+}\) conduction bands. ICB is closely analogous to its electronic (\(z = 1\)) counterpart in quantum dots and nanostructures. Coulomb blockade can be also seen in superconductors, where the charge carriers are Cooper pairs (\(z = -2e\)).

The initial (basic) ICB model for the permeation and selectivity of ion channels has recently been enhanced by the introduction of shift/corrections to allow for the singular part of the ionic attraction to the binding site (i.e. local site-binding), as well as for the effect of the ion’s excess chemical potential \(\Delta\mu\). The geometry-dependent shift of the ICB calcium resonant point resulting from these corrections leads to a change in the divalent (calcium) blockade threshold.

The model is well-fitted to describing the voltage-gated bacterial sodium channels NaChBac, NavAb, NavMs, and NvsBa. Because they are a family of relatively simple channels with discovered structures, they are widely used in modelling the general features of conductivity and selectivity.

We consider the stochastic transport of fully-hydrated ions of valence \(z\) having charge \(q = ze\) (e.g. Ca\(^{2+}\) with \(z = 2\)). As indicated above, resonant barrier-less conduction arises when the energy of the ion-site attraction \(U_{q\,SF}\) is balanced by the dielectric self-energy barrier \(U_{q\,SE}\). The ICB model allows us to derive the channel/ion parameters satisfying the barrier-less permeation conditions, and to do so from basic electrostatics taking account of charge discreteness.

The ICB balance equation can be derived using the ion’s chemical potential \(\mu\), together with the 1D quadratic form of the SF Coulomb energy \(U_{SF}\):

\[
U_{SF} = \frac{Q_{SF}^2}{2C_s} \quad Q_{SF} = \sum_i q_i + Q_f = zne + Q_f, \tag{S1}
\]

where \(Q_{SF}\) is the net charge of the SF and \(C_s\) is its self-capacitance, and \(n\) is the number of identical ions inside the SF. The sign of the charge \(q_i\) of the moving ions is opposite to that of the fixed charge \(Q_f\). Statistical mechanics tells us that, in thermal and particle equilibrium with the bulk reservoirs, the entire system has a common chemical potential \(\mu\) (the Fermi level, in other contexts). An immediate consequence is the existence of an oscillatory dependence of conduction on \(Q_f\), with two interlaced sets of singular points:

(i) Coulomb blockade points \(Q_f = Z_n\), corresponding to neutralised states of the SF, with \(Q_{SF} = 0\);
(ii) Resonant conduction points \(Q_f = M_n\), corresponding to barrier-less conduction states, with \(\Delta U_q = 0\) being the height of the energy barrier impeding passage of the ion through the channel.

The conditions for these singularities are

\[
Z_n^{ICB} = -zne \quad \text{(Blockade points)} \tag{S2}
\]
\[
M_n^{ICB} = -z(n + 1/2) \quad \text{(Barrierless points)}
\]

where \(\{n\}\) is the number of ions captured in the SF before the transition. Note the predicted independence of channel structure and dimensions, validated in earlier BD simulations.

That is the main ICB result: for \(z > 1\) the current vs. \(Q_f\) exhibits Coulomb blockade oscillations between zero-conduction blockade points \(Z_n\) at one extreme, and resonant barrier-less \(M_n\) points at the other. The oscillations in current \(I\) correspond to a Coulomb staircase in the channel/SF occupancy \(P_c\).

Fig. S6 shows the BD-simulated \(I\) and \(P_c\) for Na\(^+\) in panels (a),(b) and for Ca\(^{2+}\) in (c),(d) as functions of \(Q_f\) for different ionic concentrations, thus confirming and illustrating the main ICB phenomena. Panel (a) shows weak Na\(^+\) \((z = 1)\) conduction oscillations between resonant maxima and partially-blockaded reduced-conduction points. Panel (b) shows the corresponding smoothed-out Coulomb staircase of channel occupancy. In contrast, panel (c) illustrates the strong conduction bands observed for Ca\(^{2+}\) \((z = 2)\), i.e.
Figure S 6: Brownian dynamics simulations of multi-ion conduction and occupancy for Na\(^+\) (left column) and Ca\(^{2+}\) (right column) ions in the model of Fig. S5 as the fixed charge \(Q_f\) is varied. In (a),(b) pure Na\(^+\) baths of different concentration were used, at 10, 20, 40, 80 and 160mM as indicated; in (c),(d) pure Ca\(^{2+}\) solutions of the same concentrations were used. (a) Weak conduction oscillations in the Na\(^+\) current \(I\) (permeation events per second) can be seen. (b) The Na\(^+\) occupancy \(P\) shows a washed-out Coulomb staircase with clear evidence for concentration-related shifts. (c) The strong Coulomb blockade oscillations in the Ca\(^{2+}\) current also exhibit concentration-related shifts. The conduction bands at \(M_n\), and the blockade/neutralisation points at \(Z_n\), are discussed in the text. (d) The Ca\(^{2+}\) occupancy \(P\) forms a well-defined Coulomb staircase, again with concentration-related shifts. (Reworked from [19])

oscillations between resonant points \(M_n\) and blockaded points \(Z_n\). The corresponding occupancy plot (d) for Ca\(^{2+}\) is a well-defined Coulomb staircase in occupancy \(P_c\).

These phenomena provide for selectivity of the current. The peak conduction positions for different ionic species are shifted relative to each other, thus defining the responses of particular channels (with given \(Q_f\)) to particular ions or conditions. And vice versa, ICB oscillations (conduction bands) along \(Q_f^*\) provide a general and transparent explanation of the mutation-related transformations of selectivity [9, 13]. As illustrated, such ICB effects are expected to manifest themselves weakly for monovalent ions (e.g. Na\(^+\)), strongly for divalent ions (e.g. Ca\(^{2+}\)), and very strongly for trivalent ions (e.g. La\(^{3+}\)).

The strong oscillations in the Ca\(^{2+}\) current can serve as a basis for mapping \(Q_f^*\) onto particular mutant channels: we assume that Ca\(^{2+}\) stop bands \(Z_n\) correspond to Na\(^+\)-selective channels (non-conducting for Ca\(^{2+}\)), whereas Ca\(^{2+}\)-selective channels operate at Ca\(^{2+}\) resonant points \(M_n\) ([9, 14]).

The shapes of ICB-based Coulomb staircases for \(P_c\) are described by a Fermi-Dirac (FD) function of \(Q_f\), of the charging energy \(\Delta U_q\), or of the logarithm of concentration [14, 24]:

\[
P_c = \left( 1 + P_b^{-1} \exp \left( \frac{\Delta U_q}{k_B T} \right) \right)^{-1} \tag{S3}
\]

where the equivalent bulk occupancy \(P_b\) is related to the bulk concentration and the volume of the SF. This prediction has been confirmed directly by divalent blockade/AMFE experiments [19].

The applicability of FD statistics Eq. (S3) to classical stochastic systems obeying an exclusion principle was demonstrated rigorously by [author?]. The same FD statistics will be applied below to site protonation.

ICB (see Equation (S1) and Fig. S6) leads automatically to the strong claim that \(Q_f\) is the main determinant of selectivity in the calcium/sodium channels family. It also explains the famous and puzzling
Figure S7: Brownian dynamics simulations for Ca\textsuperscript{2+} ions in the model of Fig. S5 as the fixed charge $Q_f$ is varied, for 5 different values of $\varepsilon_p$, as shown, while keeping all other parameters fixed.

Transformations of Na\textsuperscript{+}-selective channels to Ca\textsuperscript{2+}-selective ones with increase of $|Q_f^{nm}|$ and vice versa [36, 47, 48].

Addition of the partial excess chemical potentials $\Delta\mu$ coming from different sources $Y$ [2, 45, 49] to the ICB barrier-less condition $\Delta\mu = 0$ shifts the ICB resonant points $M_n$, as described by a “shift equation” [19, 33, 34] which, for $n = 0$, is

\[ \Delta M_0 = M_0 - M_0^{ICB} = -\frac{C_s}{q} \sum Y \Delta\mu^Y. \]  

(S4)

The more important of these shifts (excess potentials) are:

- A concentration-related shift $\Delta\mu^{TS} = -k_B T \log(P_b)$ arising from the bulk entropy [45].
- A quantised dehydration-related shift $\Delta\mu^{DH}$ which we describe in section S3.4.

S3.3. Parametric tests of the model

The model is applicable to long, narrow, water-filled, channels where there is a large dielectric mismatch between the permittivities of the water and of the protein walls. Under these conditions, the electric field in the channel can be approximated as one-dimensional [50] because the field lines hardly enter the protein. The parameter range within which this approximation remains valid was explored earlier [13] in relation to the geometry (length $L_c$ and radius $R_c$) of the pore; we now consider the effect of variations in the protein permittivity $\varepsilon_p$ while holding the permittivity of the water $\varepsilon_w$ fixed at its bulk value of 80.

Fig. S7 shows the results of Brownian dynamics simulations in which the permeating current is plotted as a function of $Q_f$ for five different values of $\varepsilon_p$, while keeping all other parameters fixed, with $L_c = 10.0\text{Å}$, $R_c = 3.0\text{Å}$, and concentration 160 mM. We note that the results are relatively insensitive to large changes in $\varepsilon_p$. In particular, despite small discrepancies attributable to field penetration into the protein for $\varepsilon_p = 10$ (i.e. following a change of a factor of $5\times$), the blockade points $Z_n$ barely move, thus validating the ICB approximation.
S3.4. Resonant quantised dehydration model

Dehydration, either full or partial, is thought to be the main source of selectivity between equally charged ions, e.g. monovalent alkali metal ions \( \text{Na}^+ \) \( \text{K}^+ \) \( \text{Ca}^{2+} \). The basic ICB model takes account of hydration/dehydration only through the dielectric self-energy \( U_i^{SE} \) in a 1D Coulomb approximation \( \text{8, 8} \) which is independent of the size of the ion, so additional effects need to be included in the model.

One such effect is the discreteness of the hydration shells, which strongly influences selectivity \( \text{8, 21-23, 53, 54} \). Details of the ion-ligand interactions \( \text{53, 54} \) and multi-ion knock-on mechanisms \( \text{8, 55} \) are also important. A hydrated ion is assumed to be surrounded by spherical, discrete, single-molecule-thick water shells of equal radial thickness \( h_c \). The first shell is immediately adjacent to the ion, so the hydrated ion moves as an ion-water complex of radius

\[
R_{ion}^* = R_{ion} + h_c.
\]  

(S5)

The shell model of hydration has been well validated by experimental, analytical and numerical evidence. So, consistent with the positions of the minima observed in experimental and MD-simulated radial density functions \( \text{53, 55} \), we will take \( h_c = 0.2 \) nm for monovalent ions and \( h_c = 0.225 \) nm for divalent ions.

During their passage through the SF, ions lose/rearrange their first hydration shells, with corresponding energy penalties: while small rearrangements of the shell are relatively cheap energetically, a decrease in the coordination number immediately leads to significant expense \( \text{54} \). Generic (\( Q_f \)-independent) shell-based QD models provide a simple explanation for the difference in \( \text{K}^+/\text{Na}^+ \) selectivity between \( \text{K}^+ \) and \( \text{Na}^+/\text{Ca}^{2+} \) channels \( \text{8, 53, 54} \):

- Within narrow \( \text{K}^+ \) channels, both \( \text{Na}^+ \) and \( \text{K}^+ \) ions are fully dehydrated. The first hydration shell is more tightly-bound to the smaller \( \text{Na}^+ \) ion, \( \Delta \mu_{K}^{DH} < \Delta \mu_{Na}^{DH} \), and so the channel (counter-intuitively) favours the larger ion. Recent study has emphasised the role of direct Coulomb knock-on in the high \( \text{K}^+/\text{Na}^+ \) selectivity of \( \text{K}^+ \) channels \( \text{59} \).
- In contrast, the moderately wide \( \text{Na}^+ \) or \( \text{Ca}^{2+} \) channels accommodate both \( \text{Na}^+ \) and \( \text{K}^+ \) ions with their first hydration shells intact. Hence, \( \Delta \mu_{K}^{DH} > \Delta \mu_{Na}^{DH} \) and the channel (more intuitively) favours the smaller \( \text{Na}^+ \) ion.

\( \text{author?} \) \( \text{53} \) have suggested a simple model of QD energetics based on consideration of hydration shells as thin spherical layers, calculation of the hydration energies of Born shells, and summing over shells. The energy of the first shell \( U_1^{DH} \) is found to be

\[
U_1^{DH} \approx \frac{q^2}{8\pi\epsilon_0} \left( \frac{1}{\epsilon_p} - \frac{1}{\epsilon_w} \right) \left( \frac{1}{R_{ion}} - \frac{1}{R_{ion}^*} \right)
\]  

(S6)

In this model, an energy barrier appears due to the stripping-off of a fraction \( f_s \) of the first-shell’s spherical surface \( R_{ion}^* \), remaining outside the pore of effective radius \( R_c \):

\[
\Delta^{DH} = (1 - f)U_1^{DH}, \quad f = 1 - \sqrt{1 - (R_c/R_{ion}^*)^2}
\]  

(S7)

However, neither the generic model, nor Zwolak’s QD variant of it, consider the influence of \( Q_f \) on the selectivity sequences of a channel. To do so, we now combine the ideas of the QD models \( \text{8, 53, 54} \) with the Eisenman-inspired model of barrier-less selectivity \( \text{20, 21, 60} \) and, in particular, with the enhanced ICB model \( \text{14, 53, 54} \).

We consider an ion that retains its first shell almost untouched during its passage through the SF, so that it remains partially hydrated. In the picture proposed, the difference in \( \Delta \mu^{DH} \) is not enough per se to determine which species will be selected over the other as this choice could be changed or even inverted by the value of \( Q_f \) needed to provide barrier-less conduction, i.e. through the corresponding shift of the resonant point \( M_n \). Hence, if \( \Delta \mu^{DH} \) increases with \( R_{ion} \) then \( |M_n| \) will also increase for \( \text{Na}^+/\text{Ca}^{2+} \) channels, (though \( \text{vice versa} \) it will decrease for the narrower \( \text{K}^+ \) channels).

We assume that, for \( \text{Na}^+/\text{Ca}^{2+} \) channels, the huge difference in dielectric constant between water and protein \( \epsilon_w \gg \epsilon_p \) means that the electrostatic field \( W \) of an ion inside the channel can be decomposed in
Figure S8: Quantised dehydration scheme for monovalent (a,b) and divalent (c,d) ions in the selectivity filter (SF) of the NaChBac channel. The radius of the SF is taken as $R_c = 0.3$ nm, and the thickness of the first hydration shell as $h_c \approx 0.2$ nm. (a,c) The dehydration part of excess chemical potential $\Delta \mu^{DH}$ vs. $R_{ion}$ is shown by the blue solid line. The shaded areas indicate the range of radii where ions can fit within the SF while still retaining their 1st hydration shells intact. (b,d) The conduction current $I_{ion}$ vs. $R_{ion}$ for fixed charge $Q_f$ equal to the first resonance $M_0$ for different ions, as indicated.

In summary, after inclusion of resonant QD effects, the resultant ICB/QD model predicts the following dependences of a channel’s selectivity on $Q_f$, channel radius $R_c$ and ion size $R_{ion}$:

\[ W = W^{ICB} + W^{DH} = (1 - \kappa)W^{ICB} + \kappa W^{SP} \]

where $W^{ICB}$ is the main 1D (flat) Coulomb field localized inside the channel and the “leaking field” $W^{DH}$ is the spherical Coulomb field $W^{SP}$ of the ion $q$ attenuated by a factor of $\kappa$. The shell-based ionic free energy of dehydration, i.e. the dehydration part of the excess chemical potential $\Delta \mu^{DH}$, is calculated using the Zwolak approximation above for the energy of the first shell $U_1^{DH}$:

\[ \Delta \mu^{DH} = \kappa U_1^{DH} f_a \propto \kappa \sqrt{R_{ion}^* - R_c} \]

where $X = \{K, Na, Ca...\}$. This result describes the dehydration-related shift in the selectivity of ion channels via the shift equation Eq. S4.
Narrow channels \((R_c \approx 0.2 \text{ nm e.g. KcsA channel})\) conduct fully dehydrated ions. These channels tend to favour larger ions \((\text{K}^+)\) \((S, 54)\). When \(Q_f\) is varied, narrow channels follow the original Eisenman rule, i.e. a highly charged (“high field strength”) SF tends to favour small ions \([20]\) and vice-versa. The origin of these shifts lies in the decrease in dehydration energy with growth of \(R_{ion}\).

Moderately-wide channels \((R_c \approx 0.3 - 0.4 \text{ nm, e.g. NaChBac, NavAb, or Ca})\) conduct ions that retain significant parts of their first hydration shells. Low-charged mutants can then resonantly conduct small \((\text{Li}^+ \text{ and Na}^+)\) ions and the growth of \(|Q_f|\) leads to an inverse shift of Eisenman sequence toward the larger ions, i.e. \(\text{Na}^+ \rightarrow \text{K}^+\). This result arises from an increase in dehydration energy with growth of \(R_{ion}\), when more water molecules have to change their positions/orientations (see Eq. S9).

(author?) \([55]\) suggested an alternative (speculative) explanation of the influence of \(Q_f\) on \(\text{K}^+\) vs. \(\text{Na}^+\) selectivity in terms of a “asymmetrical snug fit” the ion to the pore. That explanation does not, however, provide an explicit dependence of the selectivity on \(Q_f\) and nor can it be tested in experiment.

S3.5. Protonation of residues in EEEE/DDDD rings

Protonation is to be anticipated in the confined space within the SF; and possible protonation of the EEEE locus has been under consideration for many years \([6, 9, 62]\).

A titration curve that defines the ionisation of residues vs. pH can be derived from exactly the same considerations as we used in our derivation of the Coulomb staircase. In the simplest case of non-interacting residues \(R_i\), the ionisation/protonation kinetics based on ionisation energy \(\Delta E_{ion}\) \([63, 64]\) results in FD statistics for the probability \(\theta\) of ionisation (and charge \(Q_f\)), usually written as a Henderson- Hasselbalch equation \([63, 65]\):

\[
\theta = \left(1 + 10^{(pK_a - pH)}\right)^{-1};
\]

\[
Q_f^* = \theta Q_f^{nn}.
\]

Taking account of the (repulsive) interaction energy \(\Delta E_{int}\) between closely-spaced charges inside the charged rings will lead to sequential increases of effective ionisation energy for the second, third and forth residues in the ring and to a composite Coulomb staircase titration curve (i.e. the sum of elementary titration curves with different \(pK_a\)) \([65, 66]\), similar to the occupancy dependence for monovalent ions like \(\text{Na}^+\), and to a shift \(\Delta(pK_a)\) in the iso-electric point of the protein.

Fig. S9 shows a sketch of the hypothesised ionisation/protonation scheme for the D191 and E191 sites, compatible with the observed selectivity and AMFE results. Both the DDDD and EEEE rings show significant interaction between charges, resulting in deviation of the titration curves from standard \(pK_a \approx 4.0\)

Figure S 9: Sketch of the putative protonation scheme for the D191 and E191 sites in NaChBac.
Figure S 10: Transformation of the Ca\textsuperscript{2+} conduction mechanism with increasing absolute value of effective fixed charge |Q\textsubscript{f}∗|, showing the Coulomb blockade oscillations of multi-ion conduction/blockade states. The neutralized states Z\textsubscript{n} providing blockade are interleaved with resonant conduction states M\textsubscript{n}. The |Q\textsubscript{f}∗| value increases from top to bottom, as shown. Green circles indicate Ca\textsuperscript{2+} ions, unfilled circles show vacancies (virtual empty states used during permeation). The right-hand column indicates the preliminary identifications of particular channels/mutants corresponding to particular mechanisms. The mutants studied here are shown in red. (Modified from [19])

Following (author?) we approximate the titration curves for the D191 and E191 residues rings as sums of four individual curves with different pK\textsubscript{a}, these putative decompositions are shown in plot (a) for DDDD and plot (b) for EEEE. Glutamate residues in the EEEE ring are significantly protonated (pK\textsubscript{a} ≈ 7) and hence the effective Q\textsubscript{f} at pH=7.4 is about −2e, whereas the DDDD ring has Q\textsubscript{f} = −3e (pK\textsubscript{a} ≈ 6). In (c) the curves sketched are composite Coulomb staircases corresponding to sets pK\textsubscript{a}(i)={4.5, 6.5, 8.5, 10.5} for EEEE and pK\textsubscript{a}(i)={4.5, 5.7, 6.9, 8.0} for DDDD. This picture provides an explanation for all the observations including, in particular, the results of divalent blockade experiments.

S4. Results

S4.1. Q\textsubscript{f}∗-mapping table

Table S 5: Divalent blockade and Q\textsubscript{f}∗-mapping. The table presents the results of divalent blockade experiments with NaChBac site-directed mutants of different nominal charge Q\textsubscript{nm}∗, such as logarithmic blockade threshold log IC\textsubscript{50}; together with estimated values of the effective charge Q\textsubscript{f}∗ and the corresponding ICB points M\textsubscript{n}, Z\textsubscript{n}.

| Mutant | Q\textsubscript{nm}∗/e | lg (IC\textsubscript{50}) | M\textsubscript{n} | Z\textsubscript{n} |
|---|---|---|---|---|
| LASWAS | 0 | 0 | 0 | Z\textsubscript{0} |
| LESWAS | -4 | -1.7 | -2 | Z\textsubscript{1} |
| LDSWAS | -4 | -2.9 | ≈-2.4 |
| LEEWAS | -8 | -4.8 | ≈-3.1 | ≈ M\textsubscript{1} |
| LEDWAS | -8 | -5.3 | ≈-3.3 |
| LEDWAS | -8 | -7 | -4 | Z\textsubscript{2} |
| LDDWAS | -8 | -8 | ≈-4.4 |

S4.2. ICB/Protonation-based conduction vs Q\textsubscript{f}∗ scheme for real channels/mutants

The protonation-related model presented above leads to a significant decrease in the effective values |Q\textsubscript{f}∗| in comparison with the nominal values |Q\textsubscript{nm}∗| of the fixed charge, and, hence to a modification of the earlier identification scheme [19]. The current scheme is built around strong Ca\textsuperscript{2+} ICB oscillations (see Fig. S6c)). It is based on the Ca\textsuperscript{2+} Z\textsubscript{n} and M\textsubscript{n} points and on the Q\textsubscript{f}∗ values obtained here with account taken of protonation.
Figure S10 presents in diagrammatic form the quasi-periodic sequence of multi-ion blockade/conduction modes arising from growth of \( n \) as \( Q_f^* \) increases, together with putative identifications of particular modes and of the NaChBac mutants used in this work. The diagram is based on the data shown in Table. S5 and Fig. S6.

- The state \( Z_0 \) with \( Q_f^* = 0 \) represents ICB for the empty selectivity filter, brought about by image forces – as observed experimentally in LASWAS (see above) and also in artificial nanopores.
- The joint \( \text{Ca}^{2+}/\text{Na}^+ \) resonant point \( M_0/M_1 \) corresponds to single-ion (i.e. \( \{ n \} = 1 \) barrier-less \( \text{Ca}^{2+} \) conduction, and can putatively be related to the OmpF porin.
- The \( \text{Ca}^{2+} \) \( Z_1 \) channel corresponds to WT LESWAS with single-ion block of the \( \text{Ca}^{2+} \) current. WT NaChBac channel conducts sodium and does not conduct calcium.
- The \( \text{Ca}^{2+} \) \( M_1 \) state corresponds to double-ion knock-on, and may be identified with the LEEWAS mutant and (putatively) with L-type calcium channel having EEEE locus and additional charged D residue in the neighbouring position.
- The LEDWAS mutant \( (Q_f^* = -4e) \) can be identified with the \( Z_2 \) \( \text{Ca}^{2+} \) blockade point, such an identification being supported both by BD simulations and by patch-clamp studies.

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