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

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Abstract

Mutation-induced transformations of conductivity and selectivity in NaChBac bacterial channels are studied experimentally and interpreted within the framework of ionic Coulomb blockade (ICB), while also taking account of resonant quantised dehydration (QD) and site protonation. Site-directed mutagenesis and whole-cell patch-clamp experiments are used to investigate how the fixed charge \(q_f\) at the selectivity filter (SF) affects both valence selectivity and same-charge selectivity. The new ICB/QD model predicts that increasing \(|q_f|\) should lead to a shift in selectivity sequences towards larger ion sizes, in agreement with the present experiments and with earlier work. Comparison of the model with experimental data leads to the introduction of an effective charge \(q_f^*\) at the SF, which was found to differ between Aspartate and Glutamate charged rings, and also to depend on position within the SF. It is suggested that protonation of the residues within the restricted space of the SF is important in significantly reducing the effective charge of the EEEE ring. Values of \(q_f^*\) derived from experiments on divalent blockade agree well with expectations based on the ICB/QD model and have led to the first demonstration of ICB oscillations in Ca\(^{2+}\) conduction as a function of the fixed charge. Preliminary studies of the dependence of Ca\(^{2+}\) conduction on pH are qualitatively consistent with the predictions of the model.

Keywords: Ionic Coulomb blockade, Ion channel selectivity, Voltage-gated sodium and calcium channels, Whole-cell patch clamp

1. Introduction

Biological ion channels provide for the highly-selective passive transport of physiologically important ions (e.g. Na\(^+\), K\(^+\) and Ca\(^{2+}\)) through the bilipid membranes of living cells. The channels consist of nanopores through complex proteins embedded in the membrane. Their selectivity for particular cations is determined by stochastic dynamics of the ions under the influence of powerful electric fields within a short and narrow selectivity filter (SF) carrying a binding site with fixed negative charge \(q_f\) \cite{1}.

Following Eisenman \cite{2}, ionic selectivity arises through a balance between repulsion by the dehydration/self-energy barrier and electrostatic attraction/affinity to the binding site. It results in resonant barrier-less conduction for the selected ion \(3\) \cite{3}, leading to selectivity phenomena such as divalent blockade of the sodium current \(3\) \cite{10} and the anomalous mole fraction effect (AMFE) \(10\) \cite{11} where the channel conductance is lower in a mixture of salts than in either of the pure salts at the same concentration.

Resonant barrier-less permeation can be described in terms of ionic Coulomb blockade (ICB) \(12\) \cite{12}, a first-principles electrostatic phenomenon that appears in low-capacitance mesoscopic systems due to charge discreteness and an electrostatic exclusion principle \(13\) \cite{13}. ICB predicts \(q_f\) 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 \(17\). ICB is closely similar to its electronic counterpart in quantum dots and nanostructures \(20\) \cite{20}, \(22\) \cite{22}. The basic ICB model for the permeation and selectivity of ion channels \(14\) has recently been enhanced \(24\) \cite{24} 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), bulk concentration, dehydration, and other sources of excess chemical potential \(\Delta\mu\). The geometry- and concentration-dependent shift of the ICB

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selectivity filter sequence

W A S W E L K E S D E -8 W A S W L D S W A S L 0 W A S -8 A 0 L S L -8 D E -4 W A S W A S 191 192 W A S L -4 D D 193 194 195 -8 E

This structure provides the highly-conserved sequence LESWAS, corresponding to residues 190 — 195). The voltage-gated bacterial sodium channels NaChBac, NavAb, NavMs, and NvsBa are a family of relatively simple channels with discovered structures that are widely used for studying the general features of conductivity and selectivity [27, 28]. Site-directed mutagenesis, varying the fixed charge $Q_f$ at the SF, is known to change their selectivity, switching sodium channels to calcium and vice versa. In turn, the alteration of Glutamate residues to similarly-charged Aspartate was also found to influence the channels’ conductivity and selectivity [25, 31]. The nature and physical origin of such transformations has remained unclear.

In this paper, we apply the ICB model to an analytical, numerical and experimental study of the effect of the fixed charge $Q_f$ on the conductance and selectivity of NaChBac bacterial sodium channels and relevant mutants [28]. Using the picture of quantised dehydration (QD), we combine the idea of quantised (shell-based) dehydration with the balanced/shift-enhanced ICB model. A systematic mutation study of selectivity in the NaChBac channel shows that the resultant ICB/QD model accounts for the experimental Eisenman sequences and for measurements of divalent blockade in the mutants. Following Eisenberg [24, 28, 31], Zhang and Shklovski [37] and Aquilella-Arzo et al [28], we take account of the effective charge $Q_f^\text{eff}$ at a channel’s binding site, which may differ from its nominal value $Q_f^{nm}$ (equal to the arithmetic sum of charges on the isolated residues). This difference is hypothesised to be due to the protonation of closely adjacent residues in the EEEE or DDDD charged rings [3, 39, 42].

2. Materials and methods

In what follows, with SI units, $\varepsilon_0$ is the permittivity of free space, $e$ is the elementary charge, $z$ is the ionic 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 the A, L, S, T and W residues are all uncharged.

2.1. Channels/mutants studied

The voltage-gated NaChBac bacterial channel [27, 28] is a tetrameric channel, whose SF is formed by 4 transmembrane segments each containing the six-amino-acid sequence LESWAS, corresponding to residues 190 — 195. This structure provides the highly-conserved {EEEE} locus E191 with a $Q_f^{nm} = -4|e|$ which is considered to create a single binding site for both mono- and divalent moving ions [27, 28]. Table I presents the set of channels generated and studied in the current research.

<table>
<thead>
<tr>
<th>Mutant channels</th>
<th>Selectivity Filter Sequence</th>
<th>$Q_f^{nm}/e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>L E S W A S</td>
<td>-4</td>
</tr>
<tr>
<td>E191D</td>
<td>L D S W A S</td>
<td>-4</td>
</tr>
<tr>
<td>E191A</td>
<td>L A S W A S</td>
<td>0</td>
</tr>
<tr>
<td>S192K</td>
<td>L E K W A S</td>
<td>0</td>
</tr>
<tr>
<td>S192E</td>
<td>L E E W A S</td>
<td>-8</td>
</tr>
<tr>
<td>S192D</td>
<td>L E D W A S</td>
<td>-8</td>
</tr>
<tr>
<td>E191D, S192E</td>
<td>L D E W A S</td>
<td>-8</td>
</tr>
<tr>
<td>E191D, S192D</td>
<td>L D D W A S</td>
<td>-8</td>
</tr>
</tbody>
</table>

Table 1: The wild-type NaChBac channel and its mutants studied in this paper, showing the amino acid sequences in their SFs and the corresponding nominal values of fixed charge $Q_f^{nm}$. The key SF positions (191,192) are shown in blue, except for charged residues which are highlighted in red. The mutants are grouped by their $Q_f^{nm}$ values. Note that for the S192K mutant the neutral serine residue is replaced by a positively charged lysine which is expected to neutralise the negatively-charged side chain of the Glutamate resulting in a $Q_f^{nm}$ value of 0.

2.2. Generation and expression of wild-type and mutant NaChBac channels

The NaChBac (GenBank accession number BAB05220) cDNA construct containing 274 amino acid residues was synthesised by EPOCH Life Science (www.eppichlifescience.com) and subcloned into the mammalian cell expression vector p’Tracer-CMV2 (Invitrogen). Single amino acid mutations in the pore region of NaChBac were generated by site-directed mutagenesis using oligonucleotides containing the sequence for the desired amino acid substitutions (primers are listed in Supplementary Material Table S1) and Q5 Site-Directed Mutagenesis Kit (New England BioLabs Inc.). All mutations were confirmed by DNA sequencing. Wild-type NaChBac and mutant cDNAs were transiently transfected into CHO cells with TransIT-2020 (Mirus Bio). Transfected cells were identified by GFP fluorescence using an inverted fluorescence microscope (Nikon TE2000-s) and used for electrophysiological investigation 24—48 hours after transfection.

2.3. Electrophysiology

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 $V_{hold}$ of -100mV. Details of the patch-clamp methods are presented in the Supplementary Material.

3. Models and theories

The features and mechanisms of electro-diffusive motion of ions through biological ion channels have been the
subject of numerous theoretical and simulation-based studies, performed with very different scales, models and methods \[1^{15}, 45\], including (in order of decreasing model detail) all-atom molecular dynamics (MD) simulations \[3\], mesoscopic Brownian dynamics (BD) simulations \[43, 44\], Monte-Carlo simulations \[45\], and Poisson-Nernst-Planck (PNP) simulations \[16\]. The different models represent different physical scales and provide complementary information. Simplified electrostatically-controlled and self-consistent BD models have already shown their utility for describing relatively wide calcium/sodium channels \[3, 16\] \[18\] \[14\] \[45\].

3.1. Self-consistent electrostatic and Brownian dynamics model

A simple electrostatic/BD model (Supplementary Material, Fig. S5) is used to describe 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 \( R \approx 0.3\) nm and length \( L \approx 1\) nm.

3.2. 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 \[13-15\] or artificial \[16, 23\]. ICB claims that for \( z > 1\) (e.g. for \( \text{Ca}^{2+}\)) the current \( I \) vs. \( Q_f \) exhibits Coulomb blockade oscillations (multi-ion conduction bands \[17\]) between zero-conduction blockade points \( Z_n = -z\epsilon n e\) at one extreme, and resonant \( M_n = -z\epsilon(n + 1) e\) points with barrier-less permeation at the other, where the index \( n \) is equal to the number of ions captured at the SF. The oscillations in \( I \) correspond to a Coulomb staircase in the channel/SF occupancy \( P_e\) (see Supplementary Material, Fig. S6); the resonant points \( M_n \) correspond to the \( n \rightarrow n + 1 \) transition in \( P_e\).

For completeness, and for convenience of the reader, the Supplementary Material provides a brief summary of the ICB model \[16, 24, 17\]. A brief description of ICB is also presented in Wikipedia \[https://en.wikipedia.org/wiki/Ionic_Coulomb_blockade\].

3.3. 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 \[3, 46, 48, 49\]. The basic ICB model takes account of hydration/dehydration only through the dielectric self-energy \( U_q^{SE} \) in a 1D Coulomb approximation \[3, 14\] 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 \[3, 5, 50, 51\]. Zwolak et al. \[50\] 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 summation over shells.

We combine Zwolak’s model with ICB, yielding the ICB/QD model which predicts that, for moderately wide \( (R_c \approx 0.3\) nm) \( \text{Ca}^{2+}/\text{Na}^+ \) channels, the growth of \( |Q_f| \) leads to a shift of Eisenman sequences toward larger ions, i.e. \( \text{Na}^+ \rightarrow \text{K}^+ \). This result arises from an increase in dehydration energy with growth of \( R_{in} \) (see Supporting Material Fig. S7). That is the main result of the ICB/QD model that will be tested experimentally here. In contrast, the narrower \( (R_c \approx 0.2\) nm) KcsA potassium channel demonstrates an inverse \( \text{K}^+ \rightarrow \text{Na}^+ \) shift with growth in field strength \[2\].

The model is described in the Supplementary Material.

3.4. Protonation of EEEE/DDDD charged rings

Protonation of charged residues is to be anticipated in the confined space within the SF. Possible protonation of the EEEE locus has been under consideration for many years \[18, 53, 46\] and the present results can be construed as additional evidence in favour of this hypothesis. Note that Boiteux et al. \[53\] have recently discussed possible alternative explanations for \( \text{Na}^+ \) selectivity in sodium channels.

The proposed protonation model is described in the Supplementary Material.

4. Results and discussion

We perform an experimental study of mutation-induced transformations of conductivity and selectivity in NaChBac voltage-gated bacterial channels, including both \( Q_f \)-varying mutations and \( Q_f \)-conserved (D=E) substitutions within the SF, in order to see whether the results could be understood within the framework of the ICB model. Increasing the value of \( |Q_f| \) was expected \[13, 27\] to lead to:

- A resonant variation of the divalent current with \( Q_f \) (ICB oscillations)
- Stronger divalent blockade of the \( \text{Na}^+ \) current, following the Langmuir isotherm.

Previous mutant studies \[27, 29, 31\] investigated a limited number of possible (D,E) combinations in the key positions 191 and 192. Our present systematic study of the possible mutants \( L_{(\text{ES}/\text{DS}/\text{EE}/\text{ED}/\text{DE}/\text{DD})} \) WAS enables us to identify the influences of both \( Q_f^{\text{in}} \) and of the D/E substitutions in positions 191 and 192, as illustrated in Table 1. We will present selectivity sequences for monovalent and divalent ions, recorded for the mutants listed above, with \( Q_f^{\text{in}} \) varied in the range \( 0 \) to \(-8\) e, and with the permutations of D and E shown in Table 1. We have also made divalent blockade measurements, providing us with the experimental information needed for application of the extended ICB model (see below) incorporating the effect of QD.

Note that whole-cell currents were used to determine the cation permeability and selectivity sequences shown in
the ICB blockaded point

...production of any kind of ions. This condition corresponds to

...the Na⁺ ports on ion channel permeability [27–29, 52]. However, figure 1), a procedure which is in line with previous re-

...to sodium currents (details below and in the caption of

...ion permeation, we report cation conductance normalised

...cells). In an attempt to exclude those effects unrelated to

...expression levels for NaChBac mutants in CHO and COS-7

...audible Na⁺ conductance normalised peak inward current magnitudes were recorded from the same cell in a Na⁺ bath solution first of Na+

...Standard error of mean) are from at least 5 cells. For I–V relationships of the presented data see

...Fig. S3 in the Supplementary Material.

...Whole-cell currents are the product of number of channels, open probability and single channel current amplitude. Thus the whole-cell current densities for different mutants can result from a wide range of factors, including some which do not reflect ion conductance through the channel pore (e.g. Yue et al. [28] report different expression levels for NaChBac mutants in CHO and COS-7 cells). In an attempt to exclude those effects unrelated to ion permeation, we report cation conductance normalised to sodium currents (details below and in the caption of figure 1), a procedure which is in line with previous reports on ion channel permeability [27, 28, 52]. However, for completeness, the Na⁺ current density for each of the mutants used in the current study is shown in Figure S0.

4.1. Ionic conductance for zero-charge mutants

...The ICB model predicts that, for an uncharged pore ($Q_f = 0$), the self-energy barrier $U_q^{E}$ should prevent conduction of any kind of ions. This condition corresponds to the ICB blockaded point $Z_0$ [13].

In the experiments, the two mutations E191A (generating LASWAS) and S192K (generating LEKWAS) were used to produce two different NaChBac mutants, each with $Q_f = 0$. They exhibited no measurable conduction of either monovalent nor of divalent cations (see Supplementary Material Fig. S2.), consistent with the ICB model. The model predicts stronger current suppression for divalent than for monovalent ions, but this distinction was below the sensitivity of our measurements and could not be tested.

Similar ICB-driven blockade was recently observed in uncharged artificial sub-nm MoS₂ nanopores, where a voltage/energy gap was found corresponding to zero ionic current for both mono- and divalent ions when the voltage across the nanopore was small [23].

4.2. Monovalent selectivity sequences of charged mutants

...Earlier studies of the E191D mutation in NaChBac [29, 30] (or, equivalently, E178D mutations in NavMs [31] and vice versa) have shown that the E=D substitution in this key position leads to a significant change in selectivity features even though there is no change of the nominal charge. The mutation E191D leads to the emergence of K⁺ conduction and to a general shift of monovalent selectivity sequences toward larger ions [30]. A similar selectivity Na⁺ → K⁺ shift was recorded for the E178D mutation in the NavMs channel [31], the E→D-related selectivity shift was also observed for the NsvBa (LDSWGS) channel [23].

Fig. 1 upper panel) presents the results of our own systematic study of the influence on monovalent ionic selectivity of E=D alterations at positions 191 and 192. The peak conductivities for (a) wild type NaChBac LESWAS and for the five mutants (b)-(f) as labelled were determined by normalising peak current magnitudes from the same cell in a Na⁺ bath solution first of Na⁺, prior to replacement of the Na⁺ by the test cation. Averages ($±$Standard error of mean) are

...positions 191 and 192. The columns of Fig. 1 (upper panel) present a compar-

...D residues alteration at position 192. The different rows correspond to

...D substitution in positions 191 and 192. The different columns correspond to E=D residues alteration at position 191: E191 for the left column and D191 for the right column. The different rows correspond to S=D residues alteration in position 192. The first row \{(a), (b)\} represents the singly-charged mutants LESWAS, LDSWAS, while the other two rows represent a $2\times2$ submatrix of the nominally doubly-charged mutants LEEWAS/LDEWAS/LDDWAS. Supplementary Material Figs. S3 and S0 show the original $I$–$V$ characteristics and representative currents, respectively, for monovalent conductance.

The columns of Fig. 1 (upper panel) present a comparison of the D191 mutants (left) with their E191 counterparts (right); results confirm that the residue in position 191 is the main determinant of monovalent selectivity in NaChBac mutants: the E191D mutation provides a significant shift towards favouring the influx of larger monovalent cations. Note that the NaK channel, possessing a

Figure 1: Experimental cation permeabilities. Upper panel: monovalent cation permeabilities, for Na⁺, Li⁺, K⁺, Rb⁺ and Cs⁺ (as labelled). Lower panel: divalent cation permeabilities, for Mg²⁺, Ca²⁺, Sr²⁺ and Ba²⁺ (as labelled). Note that no divalent current could be measured for LDDWAS. In each panel the peak inward current densities $I_{peak}$ are shown for: (a) LESWAS (wild type NaChBac) with $Q_{eff}^{m} = −4e$ and the mutant (b) LDSWAS also with $Q_{eff}^{m} = −4e$; and for four mutants (c) LEEWAS, (d) LDEWAS, (e) LEDWAS, and (f) LDDWAS each with $Q_{eff}^{m} = −8e$. In each case, normalised peak inward current magnitudes were recorded from the same cell in a Na⁺ bath solution prior to replacement of extracellular Na⁺ by the test cation. Averages ($±$Standard error of mean) are from at least 5 cells. For I–V relationships of the presented data see Fig. S3 in the Supplementary Material.
similar DDDD charged ring at the SF, is also Na\textsuperscript{+}/K\textsuperscript{+} non-selective\textsuperscript{[54]}.

Plots \{(c),(d),(e),(f)\} also show that additional E192D mutations lead to a relatively weak extra shift in the same direction.

Now we consider these phenomena in more detail.

- **LESWAS, LEEWAS, LEDWAS.** All mutants having an E191 residue present Na\textsuperscript{2+}-centred Eisenman sequences, whereas increasing the nominal total charge from \(|Q_{f}^{m}| = 4e\) for LESWAS to \(|Q_{f}^{m}| = 8e\) for LEEWAS and LEDWAS leads only to a weak increase of K\textsuperscript{+} permeability.

- **LDSWAS, LDEWAS, LDDWAS.** The D191 mutants have Eisenman sequences shifted toward favouring K\textsuperscript{+}, and the difference in \(Q_{f}\) values leads to additional permeability of large-sized ions in the LDEWAS and LDDWAS mutants which have larger \(|Q_{f}^{m}|\). This double-D LDDWAS mutant exhibits an increased shift.

The observed shift of monovalent selectivity towards larger ions can be explained by the ICB/QD model (see Sec. S3.3, Fig. S7 (a),(b)), on the assumption that the E191D mutation leads to a significant increase in the effective charge \(|Q_{f}^{e}|\), whereas further mutation of position 192 to a D (from an S or E) provides only a minor increase of \(|Q_{f}^{e}|\).

We will connect the relative sizes of the increase in \(|Q_{f}^{e}|\) to smaller protonation of the DDDD ring in comparison with EEEE\textsuperscript{[59]}.

### 4.3. Divalent selectivity sequences of charged mutants

Fig. 1 (lower panel) presents divalent cation permeabilities for the mutants studied. The peak conductivities for (a) wild type NaChBac LESWAS and for the mutant channels (b)-(e) are shown for the cations Mg\textsuperscript{2+}, Ca\textsuperscript{2+}, Sr\textsuperscript{2+} and Ba\textsuperscript{2+} (as labelled). As in the case of monovalent cations, they were normalised to the peak current recorded in Na\textsuperscript{+} bath solution. No conduction of any divalent ion could be recorded for LDDWAS (Supplementary Material Fig. S4). Similarly to the monovalent case, Fig. 1 (lower panel) can be thought as a “mutation matrix” for divalent conduction.

Whereas the wild-type LESWAS channel exhibits little divalent cation permeability, in agreement with Yue et al.\textsuperscript{[28]} and Guardiani et al.\textsuperscript{[53]}, most of the mutants show some calcium conductivity. Although less pronounced, the divalent selectivity sequences demonstrate a shift toward larger ions for both the E191D and S192D mutations. Thus the LEEWAS and LEDWAS mutants show maximal permeability for Ca\textsuperscript{2+} ions, whereas the LDSWAS and LDEWAS mutants are more Ba\textsuperscript{2+}-permeable. Although there are no comparable previous studies that systematically test divalent cation permeability of equivalent bacterial sodium channel mutants, it is noteworthy that the E178D mutant (equivalent to E191D in the present student study) of another bacterial sodium channel, NsvBA, also reports comparable permeability for Ba\textsuperscript{2+}. Furthermore, consistent with the relative divalent conductances shown for the LDSWAS mutant in Figure 1, the equivalent E to D substitution in the selectivity filter of the NsvBa channel results in a relative permeability sequence of Ba\textsuperscript{2+} = Sr\textsuperscript{2+} = Ca\textsuperscript{2+} ≥ Mg\textsuperscript{2+}\textsuperscript{[29]}. Note also that Wang et al.\textsuperscript{[53]} reported that the LDSWAS mutation of NaChBac has a significant impact on organic cation binding in the pore and is thus consistent with LDSWAS and LESWAS having distinct cation permeabilities.

Remarkably, both wild-type LESWAS (\(Q_{f}^{m} = -4e\)) and the doubly-charged (\(Q_{f}^{m} = -8e\)) LDEWAS and LDWAS mutants show strong blockade of divalent Ca\textsuperscript{2+} ions, but maintain conductance for small monovalent ions (Na\textsuperscript{+}). We connect these observations with the above-mentioned growth of \(|Q_{f}^{e}|\) for the E191D, S192E and S192D mutations, and corresponding selectivity shift, in accord with the ICB/QD model (see Sec. S3.3, Fig. S7(c),(d)). The resultant conductance oscillations have ICB blockade points \(Z_{n}\) of different-order, with smaller \(n_{1}\) for LESWAS and larger \(n_{2} > n_{1}\) for LDEWAS. The exact values of \(n_{1}\) and \(n_{2}\) will be discussed below. Note that the absence of measurable divalent current for LDDWAS is attributable to high affinity blockade of the channel by Ca\textsuperscript{2+}\textsuperscript{[52]} as illustrated in Fig. 4 and is consistent with the results previously reported by Tang et al. (2014)\textsuperscript{[52]} for the equivalent mutation in NavAb.

#### 4.4. Divalent blockade of Na\textsuperscript{+} current and \(Q_{f}^{e}\) mapping

Divalent blockade is a phenomenon of a strong attenuation of a monovalent (e.g. Na\textsuperscript{+}) current \(I_{Na}\) by micromolar extracellular concentrations of divalent (e.g. Ca\textsuperscript{2+}) ions \([Ca\textsuperscript{2+}]\), manifesting a characteristic S-shaped dependence of \(I_{Na}\) on \(log[Ca\textsuperscript{2+}]\). The phenomenon has been well-documented in calcium channels\textsuperscript{[10, 46, 57]}. Divalent blockade appears on account of the stronger affinity of the divalent ions to the binding site, so that the measurement of the divalent blockade threshold \(IC_{50}\) provides a sensitive tool of evaluating this affinity. We use it as a tool to introduce an effective value of the fixed charge \(Q_{f}^{e}\) (see below).

The ICB model predicts the Langmuir isotherm/Fermi-Dirac shape of \(I_{Na}\) vs. \(log[Ca\textsuperscript{2+}]\)\textsuperscript{[13, 20]} which is known to be consistent with typical shape of attenuation curves\textsuperscript{[58, 59]}. The ICB model also predicts a linear dependence of the blockade threshold/affinity log \(IC_{50}\) on the effective charge \(Q_{f}^{e}\)\textsuperscript{[13, 22]}:

\[
\ln(\text{IC}_{50}) = b_{Q} \frac{Q_{f}^{e} - M_{0}}{e} + b_{Q} = \frac{E_{S}}{k_{B}T},
\]

\[E_{S} = \frac{z_{C}e^{2}}{2R_{c}} = \frac{\lambda_{B}L_{c}}{2R_{c}^{2}}k_{B}T\]

(1)

where \(M_{0} = ze/2\), \(\lambda_{B} \approx 0.7nm\) is the Bjerrum length\textsuperscript{[16]}, and \(b_{Q} \approx 10\) (for model NaChBac channel having \(R_{c} = 0.3nm\), \(L_{c} = 1nm\) and the self-energy \(E_{S} = 20k_{B}T\)), is a valence- and geometry-dependent coefficient (used as fitting parameters). We cannot quantify these constants \textit{a priori}, and we will use the ICB singular points \(Z_{n}\) to invert Eq. (1) and restore the map of \(Q_{f}^{e}\) vs \(ln IC_{50}\).
Fig. 2(a) shows the results of divalent blockade experiments for NaChBac channels/mutants with \( Q_n^{nm} = -4e \) and \( Q_f^{nm} = -8e \). Bath solutions containing mixtures of Na\(^+\) and Ca\(^{2+}\) in varying concentrations were used to investigate divalent blockade and the possibility of AMFE on both the whole-cell current magnitude and current reversal voltages. The experiments started in a bath solution with 140 mM Na\(^+\) and 10 mM of \([\text{Ca}^{2+}]_{\text{free}}\), which was sequentially replaced by solutions containing increasing addition of Ca\(^{2+}\) at concentrations up to 1 mM \([\text{Ca}^{2+}]_{\text{free}}\), followed by solution in which Na\(^+\) was replaced by Ca\(^{2+}\) up to 28.7 mM of \([\text{Ca}^{2+}]_{\text{free}}\) (see details in Table S3).

The LESWAS (WT) channel does not exhibit any Ca\(^{2+}\)-dependent block of the Na\(^+\) influx [26, 28], and thus the reduced current results from extracellular Na\(^+\) being replaced with equimolar Ca\(^{2+}\) and represents the effect of substrate depletion. All other mutants show divalent blockade with systematically changing \( I_{\text{Ca}} \); it decreases by the E191D, S192E and E192D mutations. The attenuation curves \( I/N_0 \) vs. \( \log[\text{Ca}] \) for different mutants are generally parallel each to other, in accordance with the ICB-model [26].

The LDSWAS mutant shows weak divalent blockade of the Na\(^+\) current with \( I_{\text{Ca}} \approx 1\mu\text{M} \). The LEDWAS and LEEWAS mutants exhibit \( I_{\text{Ca}} \approx 10\mu\text{M} \), with LEDWAS showing an additional weak shift relative to LEEWAS. The LDEWAS and LDDWAS Ca\(^{2+}\) blockade plots were further shifted toward lower concentrations relatively to the E191 mutants. Thus, (i) the D residue provides stronger site affinity than an E in the same position, but (ii) the difference between D and E is significant for the E191D mutation, but is only minor for the E192D mutation, similar to the differences in selectivity measurements (see Fig. S1).

As mentioned above, the notion of effective charge \( Q_f^* \) for ion channels was introduced [18, 36, 37] as a fitting parameter. We now propose a \( Q_f^* \) mapping (i.e. an estimation of \( Q_f^* \) for all the mutants studied) on the basis of the affinity and positions of the ICB singular points.

Fig. 2(b) presents the inversion of Eq. 1 based on reference points \( Z_1 = -2e \) and \( Z_2 = -4e \), and using the \( I_{\text{Ca}} \) data of Fig. 2(a) to map \( Q_f^* \) for mutants, intermediate between the \( Z_1 \) and \( Z_2 \). To do so we rewrite Eq. 1 in the Newton’s interpolation form:

\[
Q_f^* = Z_1 + \frac{e}{b_Q} (\ln (I_{\text{Ca}}) - X_1); \\
\frac{e}{b_Q} = \frac{X_2 - X_1}{Z_2 - Z_1} = \frac{X_2 - X_1}{z};
\]

where \( X_i = \ln (I_{\text{Ca}}(Z_i)) \). The results presented here lead to an estimate of \( b_Q \approx 5 \), which is reasonably close to the \( a \) \( a \) priori modelled value, and corresponds well to a shorter SF: \( L_0 \approx 0.5\mu\text{m} \). The full results of the mapping are presented in Table S5.

An important feature of the ICB model is that it provides a natural explanation for the strong selectivity of particular channels (i.e. those having particular values of \( Q_f^* \)) through their identification/mapping onto particular singular ICB points. Ideally, a channel should be in a near-to-resonant \( M_n \) state for the conducted ion, while in a blocking \( Z_n \) state for the non-conducted ion. This scheme works particularly well for Ca\(^{2+}\)/Na\(^+\) valence selectivity because of the strong ICB effects observed for Ca\(^{2+}\) ions.

The resultant mapping was defined as follows (see Table S5 and Fig. 2(b)):

- **LASWAS.** Uncharged mutants do not conduct any ions, which behaviour corresponds to the \( Z_0 = 0 \) ICB point. Similar \( Z_0 \)-type electrostatic blockade was observed experimentally in MoS\(_2\) nanomipes where it was used as an evidence of ICB [23].
- **LESWAS.** The wild-type NaChBac channel conducts Na\(^+\) ions but does not conduct Ca\(^{2+}\) ions. Hence, in the ICB model, it must correspond to one of Ca\(^{2+}\) stop bands \( Z_n \). We infer that \( Q_f^* = Z_1 = -2e \). That is the first \( Q_f^* \) mapping reference point. In our model, the NaChBac channel apparently possesses an effective value \( |Q_f^*| < |Q^{nm}_f| \) significantly lower than was believed previously [28, 30] and well-correlated with protonation model. The alternative inference \( Q_f^* = Z_2 = -4e \) is not consistent with the experimental observation that LESWAS channel demonstrates lower Ca\(^{2+}\) affinity than the LDSWAS mutant.
- **LDEWAS.** Experimentally, this mutant does not conduct Ca\(^{2+}\) ions, so we assume it to be close to the next (after \( Z_1 \) for LESWAS) Cu\(^{2+}\) Coulomb blockade point \( Q_f^* = Z_2 = -4e \). This point is used as the second mapping reference point.
- **LDSWAS, LEEWAS and LEDWAS are calcium-conductive “intermediate” mutants. We infer that their \( Q_f^* \) val-
ues are proportional to $I_{C50}$ in each case, in accordance with Eq. 1 and with Fig. 2(a),(b).

- LDDWAS was mapped to $Q_f^* \approx -4.4e$ using the appropriate $I_{C50}$ value.

Fig. 3 presents ICB oscillations based on the putative $Q_f^*$ map, determined as described above, for the site-directed mutants of NaChBac. Experimental data (taken from Fig. 1) are superimposed on the multi-ion $Ca^{2+}$ conduction bands found in BD simulations. The vertical arrows indicate site-directed mutants with their respective effective fixed charges $Q_f^*$ deduced by the method described above. The model used for the BD simulations takes no account of the QD corrections, so that the overall agreement with experiment should be considered as preliminary.

Fig. 4 represents the first experimental evidence for ICB oscillations of $Ca^{2+}$ conduction in biological ion channels. The oscillations manifest themselves strongly at room temperature, unlike their electronic counterpart in quantum dots which become significant only at low temperatures. We emphasise that $Q_f^*$ constitutes the main determinant of selectivity, in agreement with the ICB/QD model.

Supplementary Material Fig. S8 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. 3.

4.5. Protonation as the putative origin of $Q_f^*$

The negative fixed charge $Q_f$ of bacterial channels and their mutants is provided by the ionised side chains of the Aspartate (D) and Glutamate (E) residues, which are the only negatively-charged protein side chains. They are characterised by their iso-electric point $pK_a$ which is the value of the hydrogen index pH providing an ionisation of 0.5, equivalent to zero net charge on the residue. Glutamates and Aspartates have the same nominal charge ($Q_f^* = -1$) and very similar $pK_a \approx 4$, but different lengths of side chain, resulting in the the EEEE-ring exhibiting a different arrangement of side chains compared to that for the DDDD-ring. This structural difference can lead to different (and potentially opposite) effects: due to the difference in local binding and to the difference in protonation and hence in the effective fixed charge $Q_f^*$.

We hypothesise that protonation is the dominant effect and that the increase of affinity corresponding to the E191D mutation is defined by significant protonation of the relatively small-radius EEEE charged ring due to overlapping of the electron and proton clouds between neighbouring residues in the ring: such effects have been studied by Furini et al. Similar effects on the $pK_a$ and protonation state of Glutamate, also due to space restriction, were calculated for the narrower KcsA channel, where $pK_a$ was shifted to $pK_a = 9.2$. The effect of Glutamate to Aspartate substitution on EEEE protonation in $Ca^{2+}$ channels was studied by Chen and Tsien. In contrast, the DDDD ring could be more ionized at physiological pH, coming closer to the full ionization of free residues. Note that the protonation-based interpretation implies that the effective $Q_f^*$ value is not an averaged “conceptual value” but, rather, that it reflects the “true” electrostatic value of $Q_f$, differing from the nominal value $Q_f^* \approx Q_f$ for an isolated residue.

Details of the protonation model are presented in the Supplementary Material.

4.6. Preliminary study of pH dependence of $Ca^{2+}$ conductance

With protonation included, the ICB model predicts a strong dependence of $Ca^{2+}$ conductance on the pH of the external solution. It had been shown previously that the pH can alter dramatically the $Na^+$ conductance for NaChBac and $Na^+/K^+$ selectivity; there are, however, no data related to the dependence of the $Ca^{2+}$ selectivity on pH.

Fig. 4 presents the results of our preliminary study of the effect of variations in extracellular pH on $Ca^{2+}$ conductance in the LESWAS and LDSWAS NaChBac channels. The results are strongly influenced by the residue type at position 191. Panel (e) shows that the inward $Ca^{2+}$ current for wild type LESWAS was small and relatively insensitive to pH changes. Note that absolute values (pA/pF) of currents are used: the points in each given plot relate to the same mutant so that normalisation is unnecessary.

Such behaviour corresponds to the position of LESWAS on the ICB conduction vs. $Q_f^*$ map (Fig. 3), i.e. to the $Ca^{2+}$ stop band for all pH, thus confirming our interpretation of LESWAS as lying at the $Ca^{2+}$ blockade point. Panel (f) for the LDSWAS mutant demonstrates a significant calcium current $I$, decreasing with growth of pH (and $Q_f^*$),

Figure 3: BD-simulated (green line) and experimental (red circles) multi-ion $Ca^{2+}$ conduction bands / fractal Coulomb blockade oscillations vs the effective fixed charge $Q_f^*$. The conduction bands in the $Ca^{2+}$ current $I$ were simulated for a pure bath with $[Ca^{2+}] = 160$ mM. Experimental conductance data are taken from Fig. 1. The $Q_f^*$ positions of the various NaChBac mutants having $Q_f^* = 0$ (LASWAS), $Q_f^* = -4e$ (LESWAS, LDSWAS), $Q_f^* = -8e$ (LEEWAS, LDDWAS, LDDWAS), calculated by fitting Eq. 4 to AMFE data, are indicated.
Figure 4: Effect of extracellular pH on Ca\(^{2+}\) currents from the NaChBac channels LESWAS (A, C, E) and LDSWAS (B, D, F). Plots A and B show original whole-cell currents from cells in response to a depolarising step to -10 mV (from \(V_{\text{hold}} = -100\) mV) in a standard bath solution (SBS) containing 100 mM Ca\(^{2+}\) at pH 6.4 (yellow), 7.4 (green) and 8.4 (blue). Plots C and D show the mean current-voltage relationships ± the standard error of the mean (SEM) for the whole-cell currents plotted in A and B. Plots E and F show the peak current densities obtained from the current-voltage relationships shown in parts C and D.

which corresponds to the decreasing-slope side of the ICB oscillation \(I_{\text{ICB}}\) vs \(Q_f^*\). Such behaviour seems to be inconsistent with our mapping (Fig. 3) in which the LDSWAS “working point” is located on the increasing-slope side. Another question, arising in connection with protonation, is possible dependence of \(Q_f^*\) on existence of captured ion. Further investigation should be performed to understand these patterns.

We note that, regardless of the mapping, the effects of pH change on the currents for LESWAS and LDSWAS are distinct, and are consistent with the protonation state of the side chains being an important factor in determining \(Q_f^*\).

4.7. Comparison with molecular dynamics simulations

Some of the NaChBac conduction data used here have also been discussed and analysed with the illumination of MD simulations \[32, 33, 56\]. The MD approach uses a combination of equilibrium simulations, Markov state modelling and meta-dynamics to show that Na\(^{+}\) permeation in NaChBac occurs through a knock-on mechanism involving two or three ions. The MD results are validated by the consistency between single channel current measurements and the currents predicted by equilibrium simulations using linear response theory. They are also in agreement with works on the homologous NavAb channel \[61\]. Guardiani et al \[32\] show that the number of Na\(^{+}\) or Ca\(^{2+}\) ions occupying the SF of NaChBac during the MD simulations can be fully justified on the grounds of the ICB model, and that aspartates and glutamates play different roles \[56\]. In particular, the ICB model is in agreement with MD simulations showing that the 3rd Na\(^{+}\) ion can occupy the SF only in a transient way to trigger the knock-on mechanism or to be bounced back into the bulk;
see also the Supplementary Discussion of Guardiani et al. 
Thus for wild type NaChBac the results from the ICB model, the MD simulations, the BD simulations, and the experiments are all mutually consistent. The recent paper by Wang et al. adds valuable insight, in that it confirms that the acidic side chains offer a flexible binding site that is exposed to the aqueous solution. Thus it is consistent with the present manuscript in proposing possible differential protonation and hence non-equivalence of the charge associated with the E and D residues at this position in the pore.

Further research will be needed to provide a full reconciliation of the ICB/QD and MD-based conduction and selectivity models for the mutants of NaChBac.

5. Conclusions

Our mutation study of conductance and selectivity in NaChBac-based channel/mutants has revealed that the E191D mutation provides a strong shift of monovalent and divalent selectivity sequences toward larger ion sizes, to-gether with a corresponding reduction in divalent selectivity sequences toward larger ion sizes, to-

The overall conclusion is that, allowing for possible protonation, the ICB/QD model provides a good description of most features of the conduction and selectivity of the NaChBac channel and its mutants. The model could also be applicable to other biological ion channels and to artificial nanopores.

Authors Contributions

OAF: Experimental design, conducting experiments, text writing and editing, data analysis.
IKK: Experimental design, model development, text writing and editing, data analysis.
WATG: Model development, data analysis
MLB: Brownian dynamics simulations for parametric testing of ICB model
DGL: Model development, data analysis
SKR: Experimental design, data analysis, text writing and editing
PVEMcC: Model development, data analysis, text writing and editing

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Appendix A. Supplementary material

Supplementary Material for this article is available online at *** [PLEASE ADD LINK]

Appendix B. Supplementary data

Supplementary Material for this article is available online at https://dx.doi.org/10.17635/lancaster/researchdata/329


