On the selectivity of the NaChBac channel: a computational analysis

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SUPPORTING INFORMATION

Supplementary Results

Time evolution of hydration and coordination of ions

An interesting information that can be retrieved from equilibrium simulations is the time evolution of the number of water molecules and protein-supplied oxygen atoms coordinating the ions inside the Selectivity Filter. In order to perform this analysis it is first necessary to define a distance cutoff to identify the oxygen atoms in contact with the Sodium and Calcium ions inside the SF. Following Ref¹⁰ the distance cutoff was defined as the position of the first minimum in the bulk ion-water oxygen radial distribution function. As can be observed in Supplementary Figure 1 the minimum between the first and second peak of the Ca²⁺-water RDF is extremely flat and wide and the limit of the first hydration can be chosen at the mid-point at 3.5 Å. Integrating the Ca²⁺-water RDF up to this point, the number of water molecules in the first solvation shell of Calcium can be evaluated around 4.5. A similar calculation performed on the Na⁺-water RDF identifies 5 water molecules in the first hydration shell whose position is located at about 3.2 Å in agreement with the value presented in Ref.¹⁰

Radial Density Functions were also computed to study the interactions of Sodium and Calcium ions with chloride (Supporting Figure 2). The Na⁺-Cl⁻ RDF shows that the position of the minimum between the first and second peak is at 3.5 Å which means that the chloride coordination shell is extensively overlapping the hydration shell of Sodium ions. However, when the RDF is integrated, it turns out that on average only 0.05 Cl^- ions reside in the first shell while 0.4 ions are present in both the second and third shell. Roughly speaking this means that in approximately half of the frames a chloride ion is in the second shell and in the remaining 50% of the trajectory the chloride ion is in the third shell while the Cl⁻ ion is hardly ever in the first shell. The Ca²⁺-Cl⁻ RDF reveals a completely different

pattern. Similarly to the Ca^{2+} -water⁻ RDF, the minimum between the first and second minimum is flat and wide and the boundary of the first coordination shell can be located at 3.5 Å. If this RDF is integrated up to this limit the number of chlorides in the first coordination shell is estimated around 1.5. This means that the first chloride coordination shell is completely overlapping with the first hydration shell and it typically contains one or two Cl⁻ ions. This result appears to be in agreement with the findings of Di Tommaso $et al^1$ who performed simulations of a Ca^{2+} ion in pure water and in aqueous alkali halide solutions. Analyzing the position of the first peak of the Ca-F and Ca-Cl RDF, these authors demonstrated that fluoride and chloride ions tend to enter into the first coordination shell. In particular, Calcium was shown to be present as $CaCl(H_2O)_6+$ in the most dilute solutions (0.11 mol/kg), as $CaCl(H_2O)_{5.4}+$ in the NaCl solution with a concentration similar to that of sea water (0.47 mol/kg), and as $CaCl_2(H_2O)_4$ in highly concentrated NaCl or CsCl aqueous solutions.

Using the distance cutoffs derived from RDF plots it is possible to follow the time course of the number of water- and protein-provided oxygens coordinating the ions in the SF (Supporting Figure 3). For both ions most of the coordinating oxygens are supplied by water. This shows that the cavity of NaChBac at variance of *e.g.* KcsA, is sufficiently wide to allow the permeating ions to retain a significant part of their hydration shell. It can also be noted that the average total number of oxygens coordinating a Sodium ion in the SF is 5.9 which is consistent with the number of water molecules belonging to the first hydration shell in the bulk. Conversely, the average total number of oxygens coordinating a Calcium ion in the SF is about 8.0, largely exceeding the 4.5 water molecules of the first hydration shell determined from the integration of the first peak of the RDF. This shows that Calcium is more hydrated in the SF of NaChBac than it is in the bulk solution so that access of the ion into the channel does not imply a desolvation penalty but rather a solvation prize. No Chloride ion was observed to access the Selectivity Filter when NaChBac was simulated either in the presence of NaCl or CaCl₂.

The plots in Supporting Figure 3 also reveal that Sodium ions on average coordinate 2 oxygen atoms provided by the protein. A closer inspection shows that on average 0.5 oxygen atoms are provided by the side chains of the glutamates of the EEEE-ring while 1.5 oxygen atoms are supplied by other protein residues. Calcium ions on the other hand, show a marked preference for negatively charged ligands either interacting with one or two oxygens typically provided by the side-chain of the same glutamate residue. On average each Ca^{2+} is coordinated by 0.96 glutamate oxygens and 0.04 oxygens provided by other protein residues.

Concentration dependence of Calcium hydration

As already discussed in the main paper, Di Tommaso *et al*¹ showed that the hydration number of calcium drops from 7 to a value between 4 and 6 as a result of the presence of varying concentrations of salts not containing Calcium. Following this line of thought it can be speculated that increasing the concentration of a Calcium containing salt like CaCl₂, calcium hydration should be affected as well. In order to test this working hypothesis we performed simulations of a system comprizing variuos numbers of calcium and chloride ions in a water box. While in the first system the water box contained a single calcium ion, in the other three systems the number of ions were chosen to yield concentrations of CaCl₂ 100 mM, 250 mM and 500 mM. A more detailed description of the simulated systems is provided in Supporting Table 3.

All simulations were performed with the NAMD $2.11b2^2$ suite of programs using the ff14SB force field³ and the TIP3P water model in conjunction with monovalent ion parameters for Ewald and TIP3P water from Joung and Cheatham.⁴ The Calcium parameters were taken from Bradbrook *et al.*⁵ The system first underwent 1000 steps of conjugate gradient minimization and it was then equilibrated for 5 ns in the NPT ensemble. Data for analysis were collected from further 20 ns of simulation using the same protocol. Pressure was kept at 1 atm by the Nose-Hoover Langevin piston method while temperature was kept at

300 K by coupling to a Langevin thermostat with damping coefficient of 1 ps⁻¹. Long-range electrostatic interactions were evaluated with the smooth particle mesh Ewald algorithm except for the case of the system containing a single calcium ion where electroneutrality was violated. For the short-range non-bonded interactions, we used a cutoff of 12 Å with a switching function at 10.0 Å. The integration time step was 1 fs, and no covalent bond was constrained.

As illustrated in Supporting Table 4, the analysis of the Calcium/Water-Oxygen Radial Density Function shows that regardless of the CaCl₂ concentration, the first peak is centered at 2.35 Å while the minimum between the first and second peak was invariably at 3.5 Å. The number of water molecules in the first hydration shell of Calcium, however, is affected by the concentration of the salt, dropping from about 7.5 in the single-calcium system, to a value between 5 and 6 in the presence of higher CaCl₂ concentrations. This pattern appears to be consistent with the observations of Di Tommaso *et al* on the influence of halkali halide solutions on calcium hydration. This behaviour can be possibly interpreted in terms of the establishment of Calcium-Chloride ion pairs whereby chloride replaces one or two water molecules decreasing the hydration number of Calcium.

Conformational flexibility of SF Glutamates

Several Molecular Dynamics studies on NavAb and NavMs highlighted a pattern of isomerization of the glutamates of the SF upon interaction with sodium or calcium ions.^{6,7} In order to check the possible occurrence of this pattern also in NaChBac we followed the time-course of the χ_2 dihedral angle of the four glutamates. The isomerization event is particularly evident in the unbiased simulation in the CaCl₂ solution where a single Ca²⁺ ion enters into the SF. The access of this ion in the SF can be traced following its distance with respect to the two O_{ϵ} atoms of the glutamates of the EEEE-ring. As illustrated in Supporting Fig 10 and 11, when the distance between the Calcium ion and at least one of the two O_{ϵ} atoms of a glutamates decreases to approximately 4.0 Å, the χ_2 dihedral angle shifts from a resting

value of about -75° to a new value of approximately 50° . From the structural point of view, when the χ_2 dihedral adopts the -75° value, the Glutamate side chains are oriented towards the extracellular bath, possibly favouring the capture of a Ca^{2+} ion. Conversely, in the 50^o arrangement, the glutamate side chains are bent toward the lumen of the SF, in the direction of the incoming ion. This behaviour allows a more detailed interpretation of the time course of the χ_2 dihedrals and of the Ca²⁺-O_e distances shown in Supporting Fig 10 and 11. In particular, it can be noted that the Calcium ion comes particularly close (~ 2 Å) to Glu51, so that the side-chain of this glutamate only need a moderate bending to follow the ion, which justifies the small new value (~ 35°) adopted by the χ_2 dihedral. By contrast, in all the other cases the distance between the incoming calcium ion and the O_{ϵ} atoms is never lower than 4.0 Å imposing a more pronunced bending of the side-chain corresponding to $\chi_2 = \sim 50^{\circ}$. In the case of Glu191(III) and Glu191(IV) the longer Ca²⁺-O_e distance weakens the interaction between the calcium ion and the glutamate side chain so that the χ_2 dihedral switches back and forth between the -75° and 50° values and actually spends a longer time in the resting position than in the "dunked" one. A partially different behaviour is exhibited by Glu191(II) where the Ca²⁺-O_{ϵ} distance is approximately 4.0 Å as in the case of Glu191(III) and Glu191(IV) but the χ_2 angle stably oscillates in the 50^o band and only seldom reverts to the -75° region. This suggests that the stability of the "dunked" conformation does not depend only on the $\mathrm{Ca}^{2+}\text{-}\mathrm{O}_{\epsilon}$ distance, but other elements such as the orientation of the side chain could be important.

The side-chains of the SF glutamates show high flexibility also when the unbiased simulation is run in a NaCl solution. As shown in Supporting Fig 12 and 13 also in this case the χ_2 dihedral can oscillate in two bands centered on -75° and 50°, however it is more difficult to study the relation between access of a Na⁺ ion and the transition from the resting to the bent conformation because in this case the SF is almost always occupied by two sodium ions and there are frequent events of access of a third ion (Fig 2 of Main Paper). The plot of the minimal distance between a sodium ion and either of the two O_{ϵ} atoms of each glutamate, reveals that in the SF there is almost always at least a sodium ion close enough to the O_{ϵ} atoms to induce bending of the Glu side-chain. It is therefore not surprising that the χ_2 angle spends most of its time oscillating in the +50° band rarely reverting to the -75° region.

Details on calculation of free energies of binding

As discussed in the main paper, within the framework of alchemical transformations free energies of binding can be computed through a double annihilation protocol based on two distinct simulations:⁸ annihilation of a sodium or calcium ion in the bulk and annihilation of an ion complexed to the SF of the channel. During annihilation the electrostatic and van der Waals potentials are rescaled as a function of a control parameter λ that is varied from zero (ion completely coupled to its environment) to 1 (completely decoupled ion). Inspired by the Weeks-Chandler-Andersen method,⁹ we first switched off the electrostatic potential in the $0 \leq \lambda \leq 0.5$ interval while the van der Waals potential was left unchanged. The van der Waals energy term was subsequently linearly rescaled in the $0.5 \leq \lambda \leq 1$ interval. The rationale of this method is that a molecular entity with dispersive attraction and charges but no core repulsion makes no physical sense since the energy does not have a lower bound. Since the core repulsion is enforced by the repulsive branch of the Lennard-Jones potential, this term must be the last to vanish.

The starting conformation chosen for Na⁺ annihilation is the final frame of the 1 ns equilibration where a single sodium ion is accommodated in the SF at the level of the EEEE-ring. This bound ion was decoupled from the environment in the first annihilation whereas in the second annihilation a randomly chosen Na⁺ ion in the bulk was vanished. Conversely, the Ca²⁺ annihilations were started from the final frame of the 105 ns unbiased NPT simulation. Also in this case the SF-bound ion and a randomly chosen bulk Ca²⁺ were independently annihilated. The radius of the constraining region was chosen as 3.0 Å and 5.0 Å for calcium and sodium respectively, considering the average distance between the ion and the center of mass (of the sidechain oxygens) of the EEEE-ring.

The results of the FEP alchemical calculations are shown in Fig 6, 7, 8. For each ion we computed not only the free energy of annihilation in bulk and in the SF but also the free energy of recoupling with its environment. The curves of the forward (annihilation) and backward (creation) transformation are well superposed, showing only marginal hysteresis. The reversibility of the transformations shows that the calculation has been performed in near-equilibrium conditions and is well-converged. The free energy of annihilation of the Na⁺ ion in the SF was 95.20 kcal/mol while the free energy of annihilation of the same ion in the bulk was 93.49 kcal/mol. The fact that $\Delta G_{annihil}^{SF} > \Delta G_{annihil}^{bulk}$ suggests that the interactions between the sodium ions and the glutamates of the EEEE-ring are stronger than the interactions of this ion with the water molecules in the bulk solvent. When sodium was annihilated in the SF, to avoid its drift during uncoupling, it was constrained within a spherical region with radius of 5.0 Å. The effect on the free energy of this loss of entropy was evaluated around 0.69 kcal/mol using the expression $-RT \ln(c_o \Delta v)$. Overall, using $\Delta G_{bind} = \Delta G_{annihil}^{bulk} - \Delta G_{annihil}^{SF} - RT \ln(c_0 \Delta v),$ the free energy of binding of the sodium ion can be evaluated as: 93.49 - 95.20 + 0.69 = -1.02 kcal/mol. In a similar way, the free energy of annihilation of Ca^{2+} from the SF was 360.60 kcal/mol while the free energy of decoupling this ion from the bulk solvent was 355.47 kcal/mol. During annihilation in the SF Ca²⁺ was constrained within a sphere with a 3.0 Å radius so that the entropic contribution to the free energy change was 1.61 kcal/mol. The free energy of binding of Ca^{2+} was computed as 355.47 - 360.60 + 1.61 = -3.51 kcal/mol.

It is noteworthy that our calculations also yield the free energy of solvation of sodium and calcium ions. In general, the free energy of solvation of an arbitrary solute can also be computed through a double annihilation protocol where, as shown in Fig 9, the solute is annihilated both *in vacuo* and in the solvated state.⁸ If $\Delta G^0_{annihil}$ is the annihilation free energy of the solute *in vacuo* and $\Delta G^1_{annihil}$ is the free energy of annihilation in solution, the free energy of solvation is yielded by: $\Delta G_{solv} = \Delta G^0_{annihil} - \Delta G^1_{annihil}$. However if, as in our case, the solute is a single ion, the free energy of annihilation in vacuum vanishes (since we are dealing with a single ion the energy of bonded interactions is zero and since we are in vacuum the energy of non-bonded interaction vanishes too) and the free energy of solvation reduces to the free energy of annihilation in solution reversed in sign. The latter quantity was already computed to estimate the free energy of binding. The free energy of Na⁺ solvation is thus -93.49 kcal/mol while the free energy of Ca²⁺ solvation is -355.47 kcal/mol. As already discussed, the much higher free energy of calcium solvation might explain the higher water coordination number of this ion in NaChBac Selectivity Filter.

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Figure 1: Ion/bulk-water-oxygen radial distribution function g(r) (black lines) for sodium (a) and Calcium (b). The minimum between the first and second peak of the RDF marks the boundary of the first hydration shell. The integral function of RDF $N_{wat} = \int_0^{min1} \rho g(r) 4\pi r^2 dr$ (green curves) in correspondence to the limit of the first shell provides the number of coordinating water oxygens.



Figure 2: Cation/Chloride radial distribution function g(r) (black lines) for sodium (a) and Calcium (b) in bulk solution. The minimum between the first and second peak of the RDF marks the boundary of the first shell of chloride coordination. The integral function of RDF $N_{Cl} = \int_0^{min1} \rho g(r) 4\pi r^2 dr$ (green curves) in correspondence to the limit of the first shell provides the number of coordinating chlorides.



Figure 3: Occupancy of Selectivity Filter. (a) number of oxygen atoms interacting with the Sodium ions in the SF; (b) number of Ca²⁺-coordinating oxygens in the SF. Blue line: water oxygens; red line: protein oxygens (not belonging to Glu); green line: glutamate oxygens; black line: total oxygens. The selectivity filter was defined as the region along the channel axis in the range $-10 \le z \le 14$ Å. The coordination analysis was performed usung cutoff distances of 3.2 and 3.5 Å for Na⁺ and Ca²⁺ respectively.



Figure 4: (A) Original traces showing whole cell NaChBac-mediated currents, recorded using PS1 and SBS-Cs. (B) Mean peak currents (normalised to currents recorded in SBS-Na; closed squares) -voltage relationships of NaChBac-mediated whole cell currents recorded in SBS-Cs (open triangles) and SBS-Na containing 7.67 mM Cs-methanesulfonate (open circles) and SBS-Na containing 79.72 mM Cs-methanesulfonate (closed triangles). Error bars represent SEM of mean values determined from at least 5 independent experiments.



Figure 5: Typical whole cell currents from CHO cells expressing L226P NaChBac mutant channels recorded using SP1 and in response to test voltages ranging from 0 mV to -185 mV (in -15 mV steps from V_{hold} of -30 mV) in the bath solution containing (A) 140 mM Na + (SBS-Na) and with Na⁺ replaced with either (B) 100 mM Ca²⁺ (SBS-Ca) or (C) 140 mM Cs⁺ (SBS-Cs). (D) Steady state whole cell current-voltage relationships from cells expressing L226P NaChBac mutant channels recorded in SBS-Na (closed circles; n=10), SBS-Ca (open circles; n=7) and SBS-Cs (closed triangles; n=5).



Figure 6: Double annihilation calculation of sodium and calcium ions in the NaChBac system. Rescaling protocol for electrostatic (black line) and Lennard-Jones energy term (red line). The λ parameter was varied through 1000 equally spaced values. For each value the system was run for 2 ps, the first 10% of which was considered equilibration.



Figure 7: Free energy change for the double annihilation of sodium ion in the NaChBac system. The λ parameter was varied through 1000 equally spaced values. For each value the system was run for 2 ps, the first 10% of which was considered equilibration. The green and magenta solid lines represent the free energy change of annihilation of the ion in complex with NaChBac and in the bulk solvent respectively. The black and blue dashed lines represent the free energy change of recoupling the ion with its environment in the SF of the channel and in the bulk solvent respectively.



Figure 8: Free energy change for the double annihilation of calcium ion in the NaChBac system. The λ parameter was varied through 1000 equally spaced values. For each value the system was run for 2 ps, the first 10% of which was considered equilibration. The green and magenta solid lines represent the free energy change of annihilation of the ion in complex with NaChBac and in the bulk solvent respectively. The black and blue dashed lines represent the free energy change of recoupling the ion with its environment in the SF of the channel and in the bulk solvent respectively.



Figure 9: Thermodynamic cycle used to compute the free energy of solvation of a generic solute. The left vertical leg represents the annihilation of the solute *in vacuo*. The right vertical leg depicts the vanishing of the solute from the solution. The lower horizontal branch describes the transfer of a non-interacting solute from vacuum to the solvent. The upper horizontal branch represents the real solvation reaction whose free energy must be determined.



Figure 10: Analysis of the χ_2 dihedral angle of the glutamates Glu191(I) and Glu191(II) (chains I ands II) of the EEEE-ring in a CaCl₂ solution. The top panels show the timecourse of the χ_2 angle (in degrees). The lower panels show the time course of the distance between the O_{ϵ} atoms of the glutamates and the single Ca²⁺ ion that accessed the SF during the unbiased simulation.



Figure 11: Analysis of the χ_2 dihedral angle of the glutamates Glu191(III) and Glu191(IV) (chains III and IV) of the EEEE-ring in a CaCl₂ solution. The top panels show the timecourse of the χ_2 angle (in degrees). The lower panels show the time course of the distance between the O_e atoms of the glutamates and the single Ca²⁺ ion that accessed the SF during the unbiased simulation.



Figure 12: Analysis of the χ_2 dihedral angle of the glutamates Glu191(I) and Glu191(II) (chains I and II) of the EEEE-ring in a NaCl solution. The top panels show the time-course of the χ_2 angle (in degrees). The lower panels show the time course of the minimal distance between a Na⁺ ion and either of the O_{ϵ} atoms of the SF glutamates during the unbiased simulation.



Figure 13: Analysis of the χ_2 dihedral angle of the glutamates Glu191(III) and Glu191(IV) (chains III and IV) of the EEEE-ring in a NaCl solution. The top panels show the timecourse of the χ_2 angle (in degrees). The lower panels show the time course of the minimal distance between a Na⁺ ion and either of the O_{ϵ} atoms of the SF glutamates during the unbiased simulation.

Table 1: Parameters of NaChbac equilibration. For each stage the force constants are listed of the harmonic restrants on backbone (BB), side-chains (SC), phospholipid heads (Lip-Head), and dihedral angles (Dihed). Force constants are expressed in kcal/mol/Å². Also shown is the time-step δt (in fs) and the duration T_{sim} (in ps) of each stage.

	stage-1	stage-2	stage-3	stage-4	stage-5	stage-6
BB	10.0	5.0	2.5	1.0	0.5	0.1
SC	5.0	2.5	1.0	0.5	0.1	0.0
Lip-Head	5.0	5.0	2.0	1.0	0.2	0.0
Dihed	500.0	200.0	100.0	100.0	50.0	0.0
δt	1.0	1.0	1.0	2.0	2.0	2.0
T_{sim}	25.0	25.0	25.0	200.0	200.0	200.0

Table 2: Total concentrations and corresponding ion activities for solutions used in the present study. Activities for Na⁺, Cs⁺ and Ca²⁺ activities ranging from 4.7, to 29.9 mM were calculated using the Debye-Hckel equation. For Ca²⁺ activities ≤ 1 mM; these were calculated using Webmaxc (http://web.stanford.edu/ cpatton/ webmaxcs.htm) and HEDTA for Ca²⁺ activities ranging from 10 μ M to 1 mM, and EGTA for activities less than 1 μ M.

Solutions (total concentrations)			Free concentrations/ion activities				
Bath	Na ⁺	Ca^{2+}	Cs^+	Chelator	Na ⁺	Ca^{2+}	Cs^+
solution							
SBS-Na	140 mM				111.9 mM		
SBS-Na	140 mM		10 mM		110.32 mM		$7.67 \mathrm{mM}$
SBS-Na	140 mM		100 mM		$105.71~\mathrm{mM}$		72.72 mM
SBS-Na	140 mM	$0.23 \mathrm{~mM}$		2 mM EGTA	110.6 mM	10 nM	
SBS-Na	140 mM	1.14 mM		2 mM EGTA	110.6 mM	100 nM	
SBS-Na	140 mM	1.86 mM		2 mM EGTA	110.6 mM	$1 \ \mu M$	
SBS-Na	140 mM	0.69 mM		1 mM HEDTA	110.6 mM	$10 \ \mu M$	
SBS-Na	140 mM	1.05 mM		1 mM HEDTA	110.6 mM	$100 \ \mu M$	
SBS-Na	140 mM	2 mM		1 mM HEDTA	110.32 mM	1 mM	
SBS-Na	140 mM	10 mM			108.22 mM	4.07 mM	
SBS-Na	140 mM	50 mM			102.06 mM	17.1 mM	
SBS-Na	140 mM	100 mM			97.3 mM	29.9 mM	
SBS-Ca	$1 \ \mu M$	100 mM			$0.717 \ \mu M$	32.6 mM	
SBS-Ca	$10 \ \mu M$	100 mM			$7.17 \ \mu M$	32.6 mM	
SBS-Ca	$100 \ \mu M$	100 mM			$71.7 \ \mu M$	32.6 mM	
SBS-Ca	1 mM	100 mM			$0.717 \mathrm{~mM}$	32.6 mM	
SBS-Ca	10 mM	100 mM			7.15 mM	32.6 mM	
SBS-Ca		100 mM	$10 \ \mu M$			32.7 mM	$6.8 \ \mu M$
SBS-Ca		100 mM	$100 \ \mu M$			32.7 mM	$68.0 \ \mu M$
SBS-Ca		100 mM	1 mM			32.7 mM	0.68 mM
SBS-Ca		100 mM	10 mM			32.5 mM	6.8 mM
SBS-Ca		100 mM				$3\overline{2.7}$ mM	

Table 3: Molecular details of the $CaCl_2$ -water simulated systems. The five columns of the Table show the $CaCl_2$ concentration, the number of calcium ions, the number of chloride ions, the number of water molecules and the length of the simulation cell respectively.

CaCl ₂ Conc (mM)	Ca^{2+}	Cl-	Water	Cell Length (Å)
Ca single	1	0	2542	46
100	5	10	2528	46
250	12	24	2507	46
500	24	48	2471	46

Table 4: Calcium hydration in the presence of different concentrations of CaCl₂. Concentrations are expressed in mM. Parameters r_{peak} and r_{shell} represent the position of the first peak and of the first minimum of the Calcium/Water-Oxygen RDF. The N_{wat} parameter represents the number of water molecule in the first hydration shell of Calcium.

Conc (M)	r_{peak} (Å)	r_{shell} (Å)	N_{wat}
Ca single	2.36	3.5	7.40
$[CaCl_2]$ 100mM	2.35	3.5	5.80
$[CaCl_2]$ 250mM	2.35	3.5	6.20
$[CaCl_2]$ 500mM	2.36	3.5	5.30

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