CONTRIBUTIONS to SCIENCE, **4** (1): 11–19 (2008)
Institut d'Estudis Catalans, Barcelona
DOI: 10.2436/20.7010.01.31 ISSN: 1575-6343
www.cat-science.cat

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The ionic selectivity of large protein ion channels

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Resum

Els canals iònics de gran amplada i de conductància alta exerceixen una funció molt important en el cicle de vida de les cèl·lules. Permeten l'intercanvi de soluts neutres i carregats a través de la membrana cel·lular i regulen l'aportació de nutrients i la sortida de deixalles. Per a realitzar aquesta funció, els canals han de discriminar entre diferents espècies iòniques. Els canals mesoscòpics permeten el transport multiiònic passiu i generalment presenten una selectivitat moderada respecte a ions positius i negatius. Aquí revisem les metodologies més usades per a estimar quantitativament la selectivitat iònica. Entre elles, la mesura del potencial elèctric necessari per a anul·lar el corrent elèctric a través del canal en presència d'un gradient de concentració, cosa que es coneix com a *potencial de corrent zero*. Assenyalem els factors clau que cal tenir en compte per a una interpretació física correcta d'aquests experiments d'electrofisiologia.

Paraules clau: electrodifusió · porus bacterials · selectivitat iònica · canal iònic · membrana biològica

Abstract

Large, highly conductive ion channels have a major functional role in the cell life cycle. They allow the exchange of charged and neutral solutes across the cell membrane envelope and regulate the influx of nutrients and the extrusion of waste products. To perform this function, channels must discriminate between different ionic species. Mesoscopic channels allow multiionic, passive transport and are usually moderately selective toward positive or negative ions. Here we review one of the most common approaches used for the quantitative estimation of channel selectivity: the measurement of the potential needed to get zero current across a channel in the presence of an electrolyte concentration gradient, also known as Reversal Potential. We highlight several key points that need to be addressed for a correct physical interpretation of these experiments in electrophysiology.

Keywords: electrodiffusion · bacterial porins · ionic selectivity · ion channel · biological membrane

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Ion channels are proteins that are embedded within the lipid bilayer membranes of cells and some viruses, where their physiological function is to regulate the passage of small charged molecules in and out of the cell or its various organelles [3,7,24]. Because of lipid dielectric properties, an ion needs 20 times more energy than an uncharged water molecule to pass through a biological membrane. This is why ion channels and pumps are necessary to transport charged molecules through cell membranes. Ion channels are mostly pore opening proteins which are fractions of a nanometre in radius and a few nanometres long. The simplest way to picture them is as hollow proteins that allow the transit of charged and neutral solutes, driven either by a concentration gradient or by the electric potential difference between the inner and the outer side of the membrane; or by both simultaneously. There are several families and sub-families of ion channels and new varieties are constantly reported. They can be classified by their permeability properties, their activation mechanism or simply by their size. However, these criteria are often interrelated. For instance, channels that perform the very specific function of translocating say, Ca^{2+} ions, display a narrow aqueous pore, while channels that allow permeation by cations and anions are usually wider. A "narrow" protein channel pore is comparable to the unhydrated size of small inorganic ions like K^+ and Na^+. This review focuses on the selective properties of wide channels, collectively known as mesoscopic channels because of their size. Transport through these channels is passive and multiionic, and it is mainly regulated by electrostatic interactions between protein ionizable residues and the permeating ions. Examples of mesoscopic channels are bacterial porins like OmpF from E. coli [14,15,38], the voltage-dependent anion channel (VDAC) of the mitochondria outer membrane [13], pore-opening toxins like the alpha-hemolysin channel secreted by *S. Aureus* [21,45] and antibiotic peptides like Alamethicin [1,11,23]. Most of them share structural motifs: for example, transmembrane pores consist largely

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of alpha-helical or beta-strand-type spanners. For a comprehensive survey of protein channels, the reader is referred to the Transport Classification Database [47]. The major functional role of these large channels in cell metabolism is to regulate the influx of nutrients and to extrude waste products, but their selectivity for low-molecular-weight inorganic ions is also relevant. In addition, they can be used to model electrostatic interactions at the nanoscale and to develop accurate theories of protein conformational dynamics where electrostatic and entropic interactions interplay.

1 Physical meaning of ion channel selectivity

Ion channel selectivity is a property that reflects the affinity of the channel for a certain type of ion. It is essential for the cell life cycle that each ionic species (especially Na^+, K^+, Ca^{2+} or Cl^-) may permeate across biological membranes at different rates, and this function is often accomplished by selective ion channels. Several experimental protocols provide different quantitative estimates of ion selectivity. Thus, conductance ratios, mole fraction (MF) experiments and reversal potential (RP) measurements define operational quantities accounting for this property [19]. These protocols provide similar but not identical results. Conductance ratio measurements yield information about the relative diffusivities of ions in a channel, whereas MF experiments quantify ion partitioning between the channel and the surrounding solution; the excess chemical potential of ions in the channel. RP is defined as the applied potential across the channel that yields zero electric current when there is a concentration gradient across the channel. RP measurement is the method of choice to quantify selectivity because it provides a joint measure of partition and diffusion. Furthermore, the sign of the measured potential provides a first estimation of the channel selectivity: For negative RP in the side of the channel with higher electrolyte concentration, the channel favours the passage of cations while in the opposite situation (positive RP) it is more permeable to anions. In the present paper we concentrate on RP as a tool for investigating the ion selectivity of membrane protein channels.

The selectivity of ion channels is a property that necessarily depends on both the channel and the electrolyte solution flowing through it. Two interdependent factors are the main contributors to the RP: diffusion effects arising from differences in ion mobilities, and electrostatic exclusion due to the interaction between permeating ions and channel ionizable residues. In addition, other factors such as steric effects related to the accumulation or rejection of ions because of their size, short-range non-electrostatic interactions, or hydrodynamic hindrance might play a role in certain cases. As many of these factors are closely related, experiments designed to separate them are necessary.

In spite of the variety of physicochemical phenomena involved, ion selectivity is often described exclusively in terms of ion concentrations and an equivalent "effective net charge" of the channel. Cation selectivity is then explained as a consequence of a negative effective charge, whereas anionic selectivity is associated with a positive one. This idea is generally accepted because most RP experiments are performed with KCl. As K^+ and Cl^- have almost equal bulk mobilities, diffusional effects are negligible and only electrostatic exclusion is relevant. However, in experiments using other electrolytes with ions of non-equal mobilities, the interaction between the channel residues and the permeating ions changes their mobilities inside the channel significantly. In such cases, the concept of "effective net charge of the channel" could be ambiguous, since the actual value of the diffusion contribution is unknown. This idea will be discussed in detail in the experimental section of this paper, where we consider RP experiments carried out with different salts of monovalent and divalent cations.

2 Methods used in modelling ion permeation

Modelling channel selectivity has a long history. Early channel models were highly simplified phenomenological approximations that retained the "essential" features of channels. However, with the advent of powerful computing techniques and the possibility of determining channel structures at atomic resolution (below 0.2 nm) the situation changed dramatically. A great number of studies focused on the rationalization of channel selectivity in terms of structure [16,29,41,42,46]. Despite the great advances in this field, computing limitations and the conditions under which channel structures are obtained (channel crystal structures are a sort of low-temperature snapshot) mean that explaining the channel selectivity observed in experiments is still a challenge. There is no definitive solution to these problems and so in this section we briefly outline, but do not evaluate, the various approaches and approximations used in modelling channel permeation. Our reasons for this are two-fold: there is abundant literature on this topic, and here we aim to give useful hints about the link between RP measurements and channel selective properties from a general point of view.

2.1 Molecular Dynamics (MD) and Brownian Dynamics (BD) simulations

When the channel protein structure is known at atomic resolution, the dynamics of the permeating ions and water molecules can be simulated provided the interaction between the mobile ions themselves and their interaction with the protein atoms is accurately modelled. Different sets of parameters commonly referred to as *force fields* have been developed to this end. In addition, several computing packages (such as, AMBER [49], CHARMM [9] and GROMACS [31]) are available to facilitate routine calculations. Unfortunately, MD is too computationally costly and reasonable simulation time (nanoseconds) is still too short when compared with the characteristic time of ion crossing across the channel (microseconds). Recent improvements in simulation techniques have made these timescales more comparable, and reliable simulations have been reported for high conductance channels [4]. Another limitation arises from the practice of applying the same atomic force constants to very different systems. Nevertheless, MD can provide useful in-

formation to be used in other approaches. In fact, it is one of the most popular tools for ion channel modelling.

Brownian dynamics simulations explicitly consider the permeating ions but regard the protein channel and the solvent as continuum structureless media [12,29]. The trajectory of each ion in the system can be obtained by solving a particular formulation of Newton's second law; the so-called Langevin equation. A major advantage of the BD approach is that it allows a direct simulation of ion-ion interactions. Since the dynamics of the water molecules and the protein atoms are no longer included and time steps can be longer, BD is many orders of magnitude faster and much less computationally costly than MD. However, it has not enjoyed great popularity, probably because it is half way between "microscopic" modelling and continuum electrodiffusion theories.

2.2 Continuum electrodiffusion models

A relatively simple way of thinking about ion movement through channels is to assume that the channel provides an aqueous medium that does not differ greatly from the aqueous solution on either side of the membrane, and that each ion moves independently of every other ion but interacts with the channel fixed charge. A mean field approximation is used for the electric force acting on every ion and Poisson equation is used for the electric potential. This approach, based on the Nernst-Planck and Poisson equations, is known in the channel literature as the PNP theory [10,17,24]. It is based on a description of the channel protein, the permeating ions and water as a continuum. When channel atomic structure is used as an input and concentration and potential at the boundaries are given, the PNP equations in three-dimensional form can be numerically solved [28]. If the 3D channel structure is not available, the 3D concentration function can be replaced by a 1D concentration (with the meaning of an average over the radial cross-section of the channel) and the whole problem becomes one-dimensional. Nevertheless, in 1D-PNP theory the equations constitute a system of coupled nonlinear differential equations that cannot be solved in closed form even in the case of a neutral channel [34]. Therefore, to obtain analytical solutions for a zero current potential, two approximations are commonly used [30]. One of them is based on the assumption of net charge neutrality throughout the diffusion region, which leads to the classical Planck expression [30]. The other is based on Goldman's assumption of a constant electric field along the diffusion zone [20], which leads to the well-known Goldman-Hodgkin-Katz (GHK) equation [25]. In the simplest case of uncharged pores the integration of PNP equations gives the following GHK potential equation:

$$RP = \frac{kT}{e} \ln \frac{D_+ C_{tr} + D_- C_{cis}}{D_+ C_{cis} + D_- C_{tr}}$$

where k, T, and e are Boltzmann's constant, absolute temperature and elementary charge, respectively; C_{cis} and C_{tr} are bulk salt concentrations on both sides of the channel, corrected with activity coefficients; and $D-$ and $D+$ are coordinate-independent diffusion coefficients for anions and cations, respectively. In channels, the ratio $D+/D-$ is regarded as a fitting parameter and it is usually represented as a permeability ratio, $P+/P-$. Despite its limitations [5,33] the GHK equation is perhaps the simplest theoretical framework for interpreting RP in a channel, and this is why it is so widely used. In fact, channel selectivity is often reported as the value of the permeability ratio instead of the measured RP. The GHK equation is useful for reporting measurements but contains little or no information about the channel structure. In the GHK approach, ion partition and ion mobility inside the channel are blended into a single permeability parameter.

2.3 Kinetic rate theories

An alternative view is to consider the channel as providing sites where the permeating ions can bind to the channel wall. Each binding site constitutes an energy well surrounded by an energy barrier. Thus, an ion moving through the channel has to cross a series of such energy barriers and energy wells as it passes from the aqueous solution on one side of the membrane, via one or more binding sites inside the channel, to the other side of the membrane. This is known as the reaction rate approach, and it is based on absolute reaction rate analysis [18]. Kinetic rate theories are a simplification of BD, rather than PNP, since they do not use mean field potentials. They are good for modelling channels with strong ion-ion interactions. Although they may help to characterize channel selectivity in some cases, it is difficult (and sometimes misleading) to relate the rate constants to real energy barriers and wells, which would make kinetic rate theories empirical models.

In this section we have provided a hierarchy of theories ranging from MD to reaction rate theories. None of these approaches is wholly satisfactory for all types of channels: each will account for some, but not all, of the channel features. Particularly, continuum electrodiffusion theories have proved very useful for modelling ion transport and selectivity in wide channels where electrostatic interactions between permeating ions and channel charged residues are the most important factors.

3 Measuring channel selectivity

3.1 Channel reconstitution in planar bilayers

Most of our understanding of channel activity comes from channel reconstitution in planar lipid membranes. This technique can provide a complete characterization of both structural and functional channel features using a tiny amount of biological material. The experimental protocols used nowadays are minor modifications of the seminal approach introduced by Mueller and Rudin [37]. A membrane forming solution is carefully smeared across a small hole drilled in an inert film separating the two compartments of a typical measuring cell. The technique is often cited as *reconstitution in black lipid membranes,* because the bilayer formation is displayed as a characteristic blackening of the film that can be followed with a simple microscope. The impermeable planar membrane that is formed represents an almost perfect insulator, which allows the detection of single ion channels once a suitable protein is incorporated into the system. One of the main problems

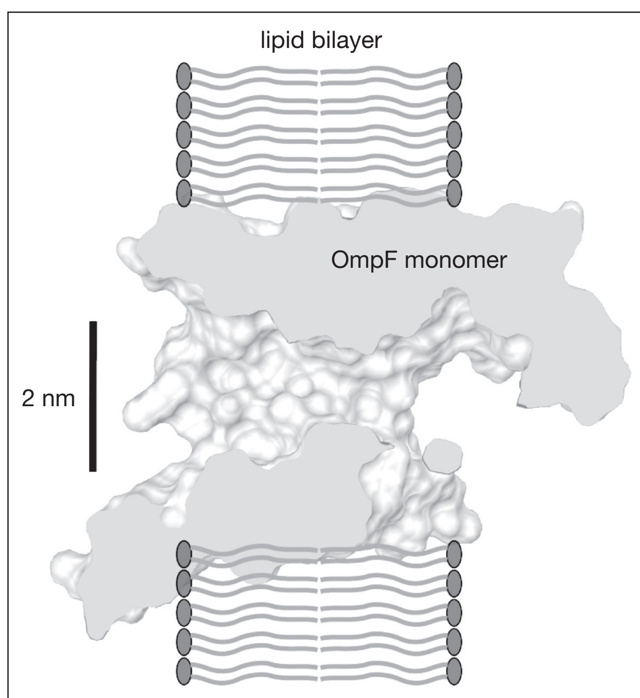

Figure 1a. Schematic representation of a protein channel reconstituted on a phospholipid planar membrane. Adapted from [6].

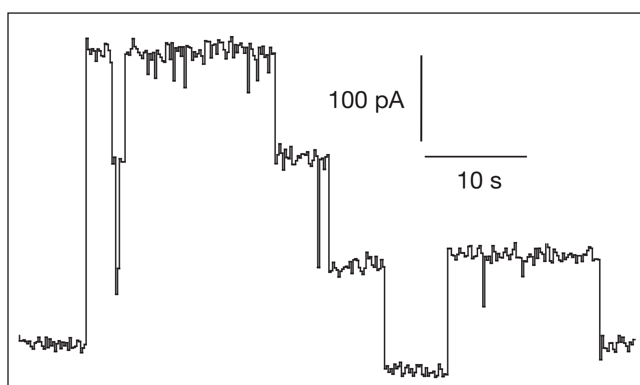

Figure 1b. Single channel current recording of OmpF porin reconstituted on a DPhPC planar bilayer. Measurements were performed at pH 7 in 0.1 M KCl and 1 mM $CaCl_2$. Adapted from [6].

with this technique is that the organic solvent still present in the bilayer may hinder the insertion of the channel-forming protein. This was successfully overcome by Montal and Mueller [36], who suggested forming the bilayer by the apposition of two lipid monolayers at an air-water interface. Instead of *painting* the bilayer, the membrane is formed by raising the level of the buffer solution after evaporation of the volatile organic solvent.

Bacterial outer membrane channels have been used extensively as model systems for other channel-forming proteins. Although they are structurally different from eukaryotic channels, the underlying transport mechanisms are usually considered to have much in common [48]. Bearing this in mind, we will explain in detail the protocol followed to functionally reconstitute the bacterial porin OmpF (Outer membrane protein F) in a phospholipid membrane. OmpF porin is a wide channel found in the external membrane of *Escherichia Coli* and characterized by its poor selectivity and almost inexistent ion specificity. The crystallographic structure of the channel reveals an asymmetric pore geometry, with relatively large entrances and a narrow constriction similar to that of general diffusion porins [5]. Wild type OmpF (a generous gift from Dr. Mathias Winterhalter) was isolated and purified from an *Escherichia coli* culture. Bilayer membranes were formed from two monolayers made from a 1% solution of diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar Lipids, Inc., Alabaster, AL, USA) in pentane on 70 to 80-µm-diameter orifices in the 15-µm-thick Teflon partition between two chambers [8,36]. The orifices were pretreated with a 1% solution of hexadecane in pentane. The total capacitance depends on the actual location of the orifices in the film (and thus on the area of the film exposed to the salt solution) but was always around 80-120 pF. Aqueous solutions of KCl and 1 mM $CaCl_2$ were buffered by 5 mM HEPES. Single-channel insertion was achieved by adding 0.1–0.3 µl of a 1 µg/ml solution of OmpF in buffer that contained 1 M KCl and 1% (v/v) of Octyl POE (Alexis, Switzerland) to 2 ml aqueous phase only on the *cis* side of the membrane while stirring. The membrane potential was applied using Ag/AgCl electrodes in 2 M KCl, 1.5% agarose bridges assembled within standard 250 µl pipette tips [8]. Potential is defined as positive when it is greater on the side of protein addition (the *cis* side of the membrane cell). Figure 1a is a schematic representation of the protein channel reconstituted on a phospholipid planar membrane. As shown in Figure 1b, the formation of OmpF channels is indicated by the characteristic step increases in the current for a given value of the applied potential. An Axopatch 200B amplifier (Molecular Devices Corp., Sunnyvale, CA, USA) was used in the voltage-clamp mode to measure the current and apply potential. Data were saved directly into the computer memory. The membrane chamber and the headstage were isolated from external noise sources with a double metal screen (Amuneal Manufacturing Corp., Philadelphia, PA, USA).

3.2 Reversal Potential measurements

RP provides a quantitative measurement of ion channel selectivity. Here we present and discuss a survey of RP experiments performed with the OmpF porin and carried out under carefully controlled pH and concentration conditions. We consider the effects of salt and pH concentration, channel orientation, lipid charge and the nature of the electrolyte. These experiments illustrate the way channel selectivity is characterized. Based on the results, in the final section we comment on the interpretation of wide channel selectivity measurements.

3.3 Salting out channel selectivity

To investigate the interaction between the channel residues and the permeating ions in detail, we first consider the OmpF channel reconstituted in a neutral lipid bilayer, diphytanoylphosphatidylcholine (DPhPC) with KCl as the electrolyte. As K^+ and Cl^- have very similar size and consequently bulk mobilities, we do not expect to observe steric effects or diffusion contributions. Figure 2 shows the measurements with KCl at a constant concentration ratio $r \equiv C_{cis}/C_{trans}$, but at different absolute concentrations (*cis* denotes the side of the protein addition and

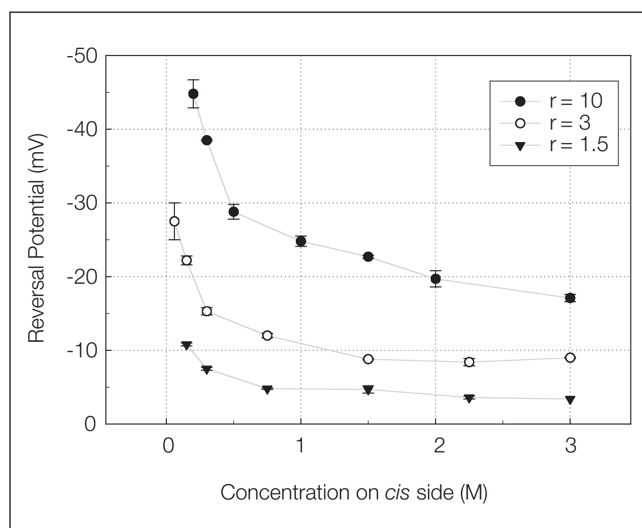

Figure 2. RP measured at three constant *cis/trans* KCl concentration ratios (10, *solid circles*; 3, *open circles*; 1.5, *triangles*) but different absolute concentrations. Membranes were formed from DPhPC at pH 6. The channel exhibits cationic selectivity: higher salt concentration on the *cis* side generates a negative RP. The absolute value of this potential decreases as salt concentration increases. Solid lines are drawn through the data points to guide the eye. Adapted from [5].

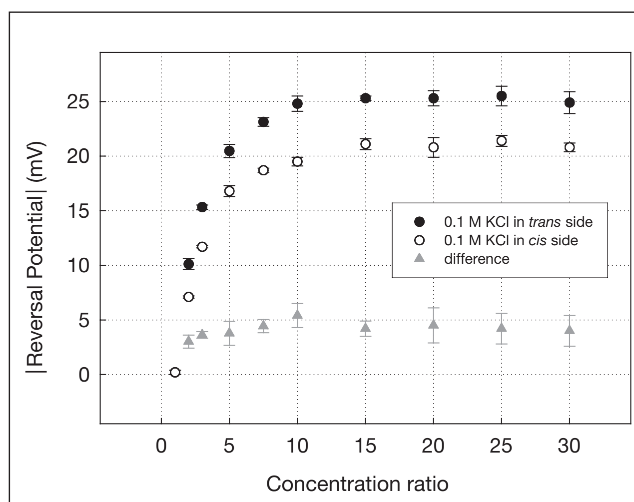

Figure 3. OmpF RP as a function of the concentration ratio for two series of measurements with oppositely directed gradients. The *filled circles* give the RP obtained from the series where $C_{cis} > C_{trans} = 0.1$ M KCl. The *open circles* are plotted as –RP for the inverse gradient: $C_{trans} > C_{cis} = 0.1$ M KCl. The channel is asymmetric: the absolute value of the RP is greater when the more concentrated solution is on the *cis* side of the membrane. The triangles represent the difference. Membranes were formed from the DPhPC at pH 6. Adapted from [5].

potential is considered positive when it is greater on this side). In these experiments both C_{cis} and C_{trans} were changed to keep their ratio at the value specified in the figure.

The data clearly show that RP is a not only a function of the concentration ratio, but also of the absolute concentration. As might be expected, concentrated solutions screen the channel fixed charges more effectively, and therefore, channel cationic selectivity is weakened. At $C_{cis} = 3$ M, the RP is reduced to less than one-half of its value at the physiological concentration.

3.4 Reversal Potential asymmetry

The OmpF channel is asymmetric both in its geometry [14] and in the fixed charge distribution [5,26]. Consequently, because of the difference in salt concentrations in the *cis* and *trans* compartments, the channel charged residues are screened differently and we should expect the RP to be sensitive to the direction of the salt gradient. In the experiments reported here, the orientation of the OmpF porin reconstituted in the lipid membrane is mostly unidirectional. This point is essential when one wants compare the measurements for a given concentration ratio (C_{cis} /C_{trans}) and its inverse (C_{trans} /C_{cis}). For a hypothetical totally symmetric channel the two experiments would give the same RP, but with opposite sign. Figure 3 shows the results of the experiments designed to examine this. In the first series, the KCl concentration ratio *r* is greater than unity ($C_{cis} > C_{trans}$) while in the second, the gradient is inverted ($C_{trans} < C_{cis}$). Salt concentration in the less concentrated solution was kept constant at 0.1 M in all cases. RP was always negative at the side with the higher salt concentration. To facilitate comparison, the two data sets were plotted together.

From Figure 3, it follows that the absolute RP value is greater by almost 25% in the orientation where the *trans* solution is kept constant at 0.1 M. This functional asymmetry probably arises from the asymmetry in the channel structure and in the fixed charge distribution.

3.5 Channel selectivity response to pH changes

One of the main goals of selectivity studies is to improve our understanding of the correlation between atomic structure and selective permeation. The experiments done at different pH (the same in both solutions) enable us to rationalize the measured RP in terms of the successive protonation and deprotonation of acidic and basic channel residues. Figure 4 shows the change of RP with pH for a 10-fold concentration ratio. The variation of RP with pH qualitatively agrees well with previously reported measurements. The anion selectivity of the channel at very acidic pH turns into almost no selectivity at pH 4 and reaches a plateau of cation selectivity in the region between pH 6 and 9. Analysis of the open channel noise and stepwise time-resolved transients in the open state demonstrate that at least three different ionization processes are involved in the pH-dependent modification of the channel transport properties. The large amount of titratable residues in the channel (102 per monomer) does not allow a simple explanation of selectivity versus pH curves in terms of one or a few pKa of charged residues. Nevertheless, detailed electrostatic calculations that take into account the interactions between residues and the protein dielectric environment provide an estimate of the total channel net charge that correlates well with the change of RP with pH displayed in Figure 4 [5,18].

3.6 Channel selectivity in different salts of monovalent and divalent cations

In this series of experiments the OmpF channel was inserted into a neutral lipid bilayer (DPhPC) and different salts of monovalent and divalent cations were used. Figure 5 shows the RP for a 10-fold concentration ratio of KCl, NaCl and two divalent salts ($CaCl_2$

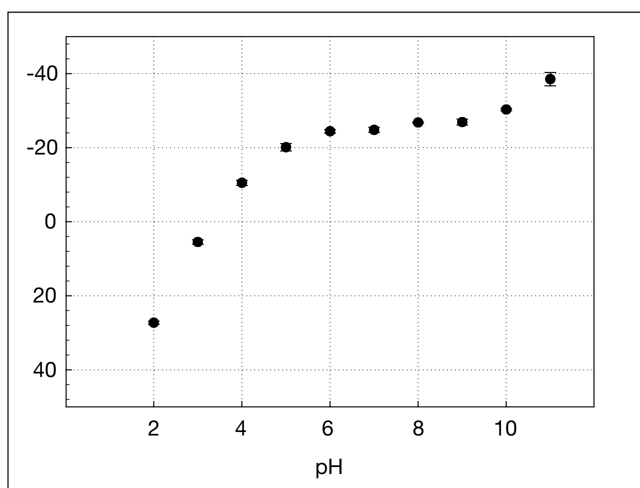

Figure 4. Dependence of RP on solution pH at C_{cis} = 1 M KCl and C_{trans} = 0.1 M KCl. Membranes were formed from DPhPC. Adapted from [5].

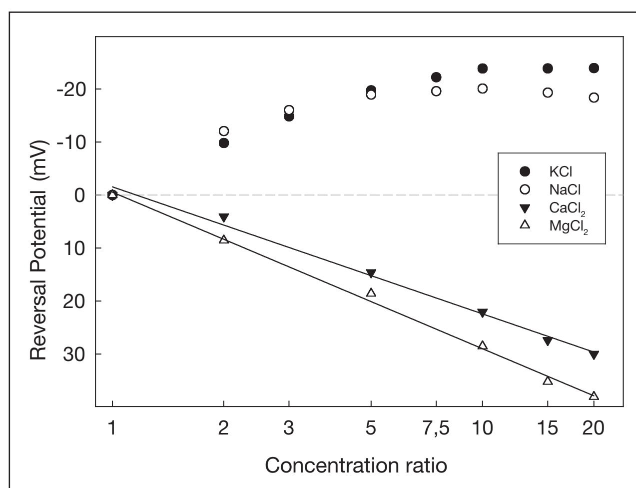

Figure 5. OmpF channel RP measured in monovalent (KCl, NaCl) and divalent ($CaCl_2$, $MgCl_2$) salts. Salt concentration on *trans* side is 0.1 M, and on *cis* side it is varied up to 20 times the *trans* concentration. The anionic selectivity displayed by the channel in the presence of 2:1 salts is mainly of diffusional origin, as shown by the good correlation of RP with the logarithm of the concentration ratio.

and $MgCl_2$) at pH 6. The comparison of salts of different cations that share the same anion could elucidate the roles of ionic exclusion, diffusion and other non-electrostatic interactions.

KCl is a suitable starting point because K^+ and Cl^- have almost equal bulk mobilities and RP is therefore dominated by electrostatic ion partition (partial exclusion of coions, Cl^-, and accumulation of counterions, K^+). Thus, concentration effects determine the shape of the curve. The sort of saturation found in the RP at high concentration ratios indicates that the channel selectivity is "salted out". In NaCl, more important diffusional effects are expected, since the bulk ion mobilities and hydrated size of Na^+ and Cl^- differ. However, RP results for NaCl reveal the same trend as those for KCl, showing that anionic exclusion dominates over other effects.

Results for salts of divalent cations are surprising. The RP sign changes, apparently inverting the channel selectivity: the channel favours anion transport and hampers cation transport, as do anion selective channels. The nature of this phenomenon is clarified in a semi-logarithmic representation: RP in $CaCl_2$ and $MgCl_2$ scale very well with the logarithm of the concentration ratio. This suggests that RP is mostly a diffusion potential, although not with ion bulk diffusivities. In fact, the apparent ratio $D+/D-$ derived from the linear fittings shown in Figure 5 is almost half the bulk value (for both 2:1 salts). The reduced effective electrical diffusivity observed for Ca^{2+} and Mg^{2+} suggests a strong interaction between divalent cations and the channel charged residues. This charge-based selectivity inversion is confirmed when experiments are performed at different pH (not shown here).

3.7 Influence of the charge of the lipid bilayer

The experiments reported above strongly suggest that electrostatic interactions are the main cause of OmpF channel selectivity. The decrease in selectivity with increasing salt concentration (Fig. 5), the selectivity asymmetry (Fig. 3) and titration upon changing solution acidity (Fig. 4) all agree perfectly with intuitive reasoning based on electrostatic considerations. Previous experiments with charged membranes show that the charge of the lipid polar groups is able to modify the surface potential significantly [35,39,40]. However, taking into account that the OmpF channel is comprised of three monomers whose size exceeds the typical Debye length of the solutions we use (~ 1 nm), it is reasonable to expect that the charge of the lipid polar groups will only have a small influence on the potential near the channel mouths.

We checked this conjecture by carrying out a series of measurements at different concentration gradients across a neutral membrane (DPhPC) and a negatively charged membrane made of diphytanoylphosphatidylserine (DPhPS). The results for KCl and pH 6 are shown in Figure 6. C_{trans} was 0.1 M and C_{cis} was varied from 0.1 M up to 3 M. The RP is always higher when the channel is embedded in the charged lipid, al-

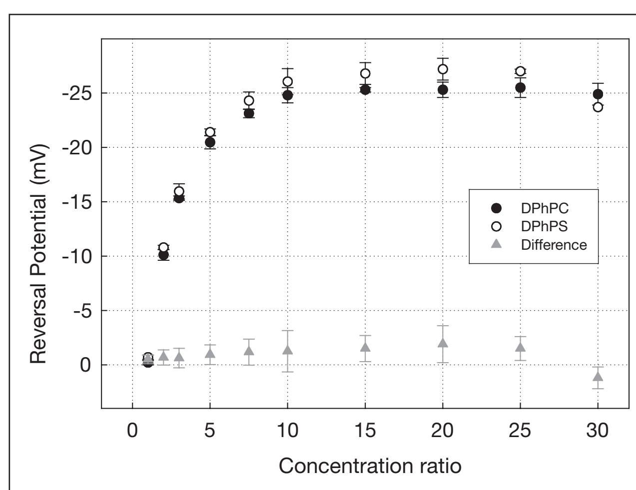

Figure 6. RP is sensitive to the lipid charge. It is larger for the negatively charged DPhPS membranes (*open circles*) than for the neutral DPhPC membranes (*filled circles*) in almost the whole range of concentration gradients at pH 6. KCl concentration on the *trans* side was kept constant at 0.1 M; the *cis* side concentration increased from 0.1 M to 3 M. Triangles represent the the difference between the two measurements. Adapted from [5].

though the difference is small (~ 5 mV). This means that the effect of the lipid charge on OmpF selectivity is measurable but does not change the channel selectivity drastically.

4 Interpretation of RP measurements

The OmpF RP measurements reported above exhibit some trends similar to those observed in other mesoscopic channels, such as alpha-hemolysin (*S. Aureus*) [27], PorA/C1 (*Neisseria Meningitidis*) [44], the mitochondrial porin VDAC (*Neurospora Crassa*) [13] and a large variety of bacterial porins [43]. These channels display common features, including: current rectification; RP asymmetry; and RP dependence on pH, concentration and electrolyte. Therefore, some interpretations of these features may be generally applicable to selectivity studies of wide channels. The basic aim of this review is to stress that ion selectivity is not an intrinsic channel property, but it is strongly dependent on several factors including, salt concentration and pH, electrolyte and lipid composition, and even channel orientation. Therefore, the interpretation of channel selectivity is necessarily linked to the particular conditions of an experiment or calculation. These conclusions can be summarized as follows:

- In contrast to the prediction of the popular GHK equation, RP is not only a function of the concentration ratio (C_{cis}/C_{trans}). It is strongly dependent on the absolute value of salt concentrations on both sides of the channel. This fact has often been overlooked and it is the basis of the range of permeability ratios obtained for a given channel and a given concentration ratio. As shown in Figure 2, the change in RP may double when it is measured at low salt concentrations, as opposed to the usual 1 M/0.1 M configuration.
- If ion selectivity were only associated with pure concentration effects (charge-based ion exclusion or accumulation) RP should be the same for all 1:1 salts. We know that this is not the case because the diffusion contribution to the measured RP is different for KCl, NaCl and LiCl, for instance. Since exclusion and diffusion cannot be split, selectivity measurements should preferably be done with KCl, whose diffusion potential (~1 mV) is comparable to the experimental error of electrophysiology experiments. As shown in Figure 5, RP values measured for salts of divalent cations have the opposite sign to those measured for 1:1 salts. In other words, the OmpF channel displays an apparent anionic selectivity for 2:1 salts. Experiments performed with $CaCl_2$ and $MgCl_2$ illustrate a case where a new type of interaction between channel residues and mobile ions is present. Chemical binding [32] or any other short-range interaction [22] completely changes the measured RP and, if it is not taken into account, may lead to channel mischaracterization. Thus, the second message is that channel selectivity should be measured with different types of salts in order to exclude any interaction that is not Coulombic.
- Asymmetry is a common feature of many protein ion channels. It may be due to pore geometry or fixed charge distribution. Although the influence of channel asymmetry in current rectification has been widely reported [6], studies of the effects of such asymmetry on channel selectivity are scarce [2,5]. In some cases, the direction of the salt concentration gradient may be very important, as illustrated by Figure 3. Thus, proper interpretation of RP experiments requires information about the directionality of the channel insertion in the lipid membrane and also about its orientation *in vivo*.
- In wide channels, selectivity is mainly determined by the interaction of the mobile ions with the charged residues lining the channel pore. (Other residues not accessible to permeant ions may also play a role.) Since ionization equilibrium of these residues is pH dependent, controlling the pH of the solutions is essential for the reproducibility of the RP measurements. In addition, RP measurements performed over a wide pH range may provide valuable information about the channel residues that are accessible to the permeating ions. This technique, combined with site-directed mutagenesis, can give clues about the channel tertiary structure when other techniques (X-Ray diffraction, NMR) do not provide such structural information.
- Finally, it is important to stress that selectivity experiments are directly comparable only when they follow the same experimental protocol. As explained in previous sections, several different techniques are available for ion channel reconstitution in lipid bilayers. Each technique involves particular details of the biochemistry (protein purification and storage, lipid composition, solvents), the electrochemistry (cell, electrodes) or the electronics (signal amplification, electrical isolation). Among these factors, the lipid composition seems to be crucial. Many selectivity measurements are performed in channels reconstituted on bilayer membranes where the lipid surface charge density is not known *a priori*. This poses a problem for the correct interpretation of RP measurements, since they depend on the lipid membrane surface charge. In the case we report here, the difference between RP in channels reconstituted on neutral and charged membranes is small (because of the size of the protein channel) but in other wide channels the effect is not negligible and should be considered [1]. Therefore, selectivity measurements should be performed in membranes of known charge density.

Acknowledgements

This work was supported by the Spanish Ministry of Education (project FIS2007-60205).

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