Permeation Through the Calcium Release Channel of Cardiac Muscle

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ABSTRACT Current voltage (*I-V*) relations were measured from the calcium release channel (CRC) of the sarcoplasmic reticulum of cardiac muscle in 12 KCl solutions, symmetrical and asymmetrical, from 25 mM to 2 M. *I-V* curves are nearly linear, in the voltage range ±150 mV ≈ 12*kT/e*, even in asymmetrical solutions, e.g., 2 M || 100 mM. It is awkward to describe straight lines as sums of exponentials in a wide range of solutions and potentials, and so traditional barrier models have difficulty fitting this data. Diffusion theories with constant fields predict curvilinear *I-V* relations, and so they are also unsatisfactory. The Poisson and Nernst-Planck equations (PNP) form a diffusion theory with variable fields. They fit the data by using adjustable parameters for the diffusion constant of each ion and for the effective density of fixed (i.e., permanent) charge $P(x)$ along the channel's "filter" (7-Å diameter, 10 Å long). If $P(x)$ is described by just one parameter, independent of x (i.e., $P(x) = P_0 = -4.2$ M), the fits are satisfactory (RMS error/RMS current = 6.4/67), and the estimates of diffusion coefficients are reasonable $D_K = 1.3 \times 10^{-6}$ cm^2/s, $D_{Cl} = 3.9 \times 10^{-6}$ cm^2/s. The CRC seems to have a small selectivity filter with a very high density of permanent charge. This may be a design principle of channels specialized for large flux. The Appendix derives barrier models, and their prefactor, from diffusion theories (with variable fields) and argues that barrier models are poor descriptions of CRCs in particular and open channels in general.

INTRODUCTION

The calcium release channel (CRC) of the sarcoplasmic reticulum of striated muscle is a complex, interesting, and important channel through which calcium ions flow from their storage site (in the sarcoplasmic reticulum) to their active site on the thin filament (Melzer et al., 1995). The channel controls and "catalyzes" this flux (from place to place) much as an enzyme catalyzes flux from reactant to product (Moczydlowski, 1986; Eisenberg, 1990; Andersen and Koeppe, 1992). In the cardiac CRC, gating is governed by the calcium concentration near part of the channel protein (Fabiato, 1983; Wier, 1990). In the skeletal CRC, gating is controlled allosterically by a protein in a neighboring membrane, the dihydropyridine receptor of the T-tubular system, which responds to the potential across the T membrane (Rios and Pizzaro, 1991; Schneider, 1994). The skeletal CRC is also regulated by Ca^{2+}-dependent mechanisms (Coronado et al., 1994; Meissner, 1994). Despite the similarity of the proteins, the gating of the cardiac and skeletal CRCs is quite different, making the CRC a subtle and interesting, as well as important and complex system (Coronado et al., 1994; Meissner, 1994). This paper describes the permeation of KCl ions through the cardiac CRC; similar experiments on other monovalent ions and on skeletal CRC are well under way.

The mechanism of permeation of ions through the CRC has received considerable attention (Williams, 1992; Coronado et al., 1994; Meissner, 1994). The CRC displays an unusually large ion conductance for monovalent cations (~750 pS with 250 mM K^+ as the current carrier) and divalent cations (~150 pS with 50 mM Ca^{2+}). The current-voltage (*I-V*) relations of (single open channels of) CRC are surprisingly linear, even when measured from −150 to +150 mV, even when the solutions bathing the channel are strikingly different. Traditional models of permeation, widely used in channology (Hille, 1975; Hille and Schwartz, 1978; Eisenman and Horn, 1983; Läuger, 1991; Andersen and Koeppe, 1992; Hille, 1992), describe ionic trajectories as a series of jumps over barriers. They imagine that ions move through a channel's pore by hopping over barriers, without collisions with other atoms. However, "there is now rather direct evidence that diffusion in dense fluids does not occur by individual molecular 'jumps' over distances of the order of a molecular diameter" (Tyrrell and Harris, 1984). (The textbook of Berry et al. (1980, p. 845) explains why diffusion occurs by hopping in gases but not in liquids. Hopping can occur in gases because they are mostly empty space. It cannot occur in liquids because they are condensed phases with little empty space: "[T]he principal difference between a dilute gas and a liquid is. . . the multiplicity of simultaneous interactions in the liquid. In a dilute gas a typical molecule is usually outside the force fields of all other molecules and only occasionally in the force field of one other molecule [during a] binary collision, whereas in a liquid a typical molecule is usually within the force fields of, say, 10 nearest neighbor molecules and is never completely free of the influence of other molecules.")

Barrier/hopping models have been applied to CRCs (Tinker et al., 1992), but they have several difficulties (see Appendix). Practically speaking, barrier models naturally predict an exponential dependence of current on voltage: N

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large barriers, of height W_j, produce currents that are more or less sums of exponentials of the form $\sum_j^N \alpha_j \exp(W_j e/kT)$. It takes a large N to describe a roughly linear *I-V* relation, from −80 to +80 mV, some $6kT/e$ (Tinker et al., 1992). When the potential range is extended to $12kT/e$ (i.e., ±150 mV), exponential functions vary over a range of some $e^{12} \cong 1.6 \times 10^5$, and their sums become awkward (although possible) descriptions of roughly linear *I-V* relations.

Another description of permeation uses diffusion theories (Goldman, 1943; Hodgkin and Katz, 1949; Levitt, 1982, 1984, 1985, 1986, 1987; Cooper et al., 1985, 1988a,b; Chiu and Jakobsson, 1989; Barcilon et al., 1993; Eisenberg et al., 1995), which give nonlinear *I-V* relations in their traditional form as "constant field" theory (Goldman, 1943; Hodgkin and Katz, 1949; discussed and derived in Chen et al., 1992, 1995b, 1997; Tang et al., 1997). Diffusion theories have often been considered crude macroscopic approximations, but recent work (Barcilon et al., 1993; Eisenberg et al., 1995) clarifies their atomic basis and shows (rigorously, using mathematics alone) that simple differential equations—which might seem to be macroscopic approximations but are not—can describe the statistical properties of the flux of discrete ions overa potential barrier of any shape. (Differential equations with continuous independent and dependent variables are commonly used (Feller, 1957, 1971; Karlin and Taylor, 1975, 1981) in the theory of stochastic processes (e.g., the probability theory of Brownian motion of particles and atoms) to describe the movement (i.e., the probability or other statistics of trajectories like flux or mean first passage time) of discrete particles and atoms.) These differential equations can be solved analytically and then, in many cases, simple integrals can describe the flux (or its rate constant), the contents of the channel, and the (conditional) mean first passage times of individual ions (see Appendix, Eq. 4).

If ions diffuse over a high barrier, the flux is described by an exponential expression (see Appendix, Eq. 5), long known (Kramers, 1940; Chandler, 1978) and experimentally tested (Fleming et al., 1986; Schroeder and Troe, 1993) in the chemical literature. The same exponential expression (Eq. 5 of the Appendix) is apparently used throughout the chemical literature. Hänggi et al. (1990) wrote the historic definitive review of the chemical literature, which cites some 700 references. Fleming and Hänggi (1993) review the more recent literature.

The high barrier expression of the chemical literature, including its prefactor, display the dependence of flux on the partition function or entropy of activation (Robinson and Holbrook, 1972; Chandler, 1978; Hynes, 1985, 1986; Berne et al., 1988). It also displays the dependence of the entropy of activation (and flux) on the underlying physical parameters of the channel and permeating ion, namely, on the diffusion coefficient of each ion, on the length of the channel, on the temperature, and on the height of the potential barrier (see Appendix, Eqs. 5 and 6).

In contrast to the expression of the chemical literature, the "Eyring" high barrier expression (of traditional barrier models of open channels; Hille, 1992) does not display the dependence of activation entropy on the underlying physical parameters. It uses a prefactor, kT/h, that is independent of physical parameters (except temperature) and an exponent that is implicitly assumed (in the papers we know of) to vary only as the potential energy varies. Thus traditional barrier models are likely to give misleading results if used to compare experiments in which the prefactor (i.e., activation entropy) or diffusion coefficient is likely to change, e.g., experiments involving different ions (with different diffusion coefficients, in all likelihood, and thus different prefactors and activation entropies); experiments with mutated or modified channels (which are likely to have modified potential barriers and thus modified prefactors and activation entropies); and experiments with different concentrations of ions (which are likely to shield fixed charge differently, have different potential barriers (Eisenberg, 1996), and thus have different prefactors and activation entropies).

Traditional barrier models have quantitative difficulties as well in describing currents found in most open channels (Conley, 1996a,b, 1997) because the diffusion coefficients that they ignore have large effects: friction reduces the flux substantially in a condensed phase, as one might expect in a system with little empty space, like a liquid (Berry et al., 1980) or protein (McCammon and Harvey, 1987; Brooks et al., 1988). When the correct prefactor is used, flux is reduced by a factor of $\sim 2 \times 10^4$ (for K^+ in the CRC channel, as we shall see), if the barrier height is held constant. When the correct prefactor is used, the barrier height must be reduced by $10kT/e \approx \log_e 2 \times 10^4$ to produce the same flux, namely the current observed experimentally. Thus fitting experimental data with a traditional channel model is impossible, if the correct prefactor is used, because barriers must be larger than, say, $\sim 3kT/e$ if the traditional model is to make any sense. A traditional model, using the correct prefactor, and the dimensions and diffusion coefficient for CRC that we report here, predicts a conductance of some 2 picosiemens, in 100 mM KCl, if the barrier is a parabola $3kT/e$ high, compared to the hundreds of picosiemens we measure under those circumstances.

Diffusion and barrier theories both include the effects of the electrical potential within the channel. The electric field (i.e., potential profile) depends on all charged species, those in the solutions, those that form the channel protein, and those that support the membrane potential. Most of these change as bath concentrations and membrane potential are changed, and so it is necessary (Eisenberg, 1996) to compute (or measure) the electric field (i.e., the profile of electrical potential) under each experimental condition, that is to say, at each transmembrane potential and in each pair of bathing solutions, as is done, for example, in the Poisson-Boltzmann, Gouy-Chapman, and Debye-Hückel treatments of electrochemistry. Otherwise, the assumed profile of potential will be inconsistent with the charges present in the system.

The Poisson-Boltzmann theory (Davis and McCammon, 1990; Honig and Nichols, 1995) is not a useful description

of open channels, because it assumes zero flux. In its place, we use a self-consistent combination of the Poisson equation of electrostatics, and the Nernst-Planck equation of electrodiffusion, that allows current to flow. Combined, the Poisson and Nernst-Planck equations form probably the simplest self-consistent generalization of the Boltzmann factor of classical channology (e.g., p. 12 of Hille, 1992) or of Poisson-Boltzmann theory to nonequilibrium situations. This system of equations is nearly the same as the drift-diffusion theory used to describe the movement of charged particles in many physical systems (Ashcroft and Mermin, 1976; Sze, 1981; Selberherr, 1984; Mason and McDaniel, 1988; Rouston, 1990; Spohn, 1991; Balian, 1992; Chen et al., 1992; Lundstrom, 1992; Chen and Eisenberg, 1993a; Mahan, 1993; Jerome, 1995). Chen and Eisenberg, (1993a) derived and applied these equations to channels and called them PNP to emphasize the importance of the Poisson equation. (PNP is apparently the first self-consistent theory (i.e., one in which the potential profile is computed from the charges present) of channels; it is certainly not the best, and probably not the last. A three-dimensional theory that includes atomic detail, short-range electrostatic forces and other chemical interactions, stochastic behavior, single filing, and dehydration/resolvation phenomena at ion entry would clearly be much better.) The PNP equations describe the electric field, the probability of location (which we call the concentration), and current flow of ions through the open channel.

In the present work, the single-channel currents of the cardiac CRC were recorded in KCl solutions, using the planar lipid bilayer method. Although Ca^{2+} movement through CRC is of the greatest importance, we chose to use K^+ as the main current carrier because calcium ions have complex effects on both permeation and gating (Tinker et al., 1992; Tripathy and Meissner, 1996).

Analysis of single-channel currents with the PNP equations indicates that the *I-V* relations of CRC (in KCl solutions ranging from 25 mM to 2 M over a voltage range of -150 to $+150$ mV) might arise from a channel with a (spatially) uniform density of (effectively one-dimensional) permanent charge in its selectivity filter. If the channel is described in more detail (i.e., if the permanent charge $P(x)$ is described by four parameters), the *I-V* relations are fit better. More experimentation is needed to justify the more elaborate description.

The Appendix discusses the difficulties of barrier models and suggests, in view of these, that they have outlived their usefulness as models of open channels.

THEORY AND METHODS

Theory

A combination of the Poisson equation of electrostatics and the Nernst-Planck equations predicts the current flow through the open channel (for given membrane potentials and pairs of concentrations of permeant ions) while simultaneously predicting the shape of the electric field. The theory is described in detail in an expository article (Eisenberg, 1996) and derived in the original papers: Eisenberg et al. (1995) provide a stochastic derivation of the Nernst-Planck part of the theory; Chen et al. (1992) and Chen and Eisenberg (1993a) derive the electrostatics.

The predictions of the PNP theory depend on the atomic structure of the selectivity filter of the channel, which is rarely known at all, and hardly ever in sufficient detail on the time scale of permeation. The theory in its practical application must then describe the channel in an approximate way, by its length and diameter, and (most importantly) by its (spatially and temporally averaged) effective one-dimensional profile $P(x)$ of permanent (i.e., fixed) charge. (Permanent charge is the charge on an atom when the nearby electric field is zero. It is the largest charge (usually by far) on an atom involved in a polar or ionic chemical bond, even in a strong electric field. Permanent charge does not include the mobile ions in the channel's pore, or the dielectric (i.e., polarization) charge induced by the electric field. Permanent charge is the intrinsic charge determined by the quantum mechanical properties of the atoms and molecule, and is tabulated for all atoms of amino acids in standard programs describing the molecular dynamics of proteins, e.g., CHARMM and MOIL (Brooks et al., 1983; Elber et al., 1993). Surprisingly few atoms in proteins have negligible permanent charge according to the "look-up" tables of these standard programs.)

Fortunately, it is simple to solve the PNP equations numerically, once one knows how, although learning how was not simple. Duan Chen has developed a rapid, stable, and accurate numerical method (Chen et al., 1992, 1997; Chen and Eisenberg, 1993a; Jerome, 1995), and the code for implementing that method is available to anyone who requests it. (The same method was previously discovered by semiconductor physicists and is presented in texts (Lundstrom, 1992; Jerome, 1995). Chen's program is written in FORTRAN 77 and has been compiled, and runs easily on a number of systems. A Windows '95 version is available from Steve Traynelis of the Department of Pharmacology, Emory University (Atlanta, GA). Both versions can be picked up (with instructions and examples) by anonymous FTP from location/pub/Eisenberg/PNP at alexandria.rpslmc.edu (i.e., IP address 144.74.3.21) or through the Internet on the World Wide Web at location http://aix550.phys.rpslmc.edu/pnp.html) (i.e., IP address http://144.74.27.66/pnp.html).)

The inputs of either version of PNP are the concentrations and potentials in the baths; the length, diameter, and dielectric constant of the channel's pore; the diffusion coefficients of permeable ions in the channel; and the permanent charge density $P(x)$. If $P(x)$, etc., were known experimentally, this program would then predict the *I-V* relations of the open channel in all solutions.

Determining parameters

Too little is known about the structure of the selectivity filter of CRC (or most other channels) to determine the permanent charge density in one or three dimensions, and too little is known about the internal dielectric properties, ionization state, or dynamics of atoms within a protein (e.g., their electrical interaction with the ions in the channel's pore) on the time scale of permeation to determine the effective charge density (relevant to permeation), even in those channels for which the crystal structure is known. For this reason, the effective charge profile $P(x)$ must be estimated by fitting the PNP theory to the experimental data itself, i.e., by minimizing the sum of the squared deviation between experimental data and theoretical prediction.

Of course, the charge profile estimated this way is not as well determined as we would wish; we would like to know that charge profile in atomic detail, on the time scale of permeation. But in a certain sense that detail is not relevant to the *I-V* curves reported here. Those curves can be predicted, as we shall see, by just a few parameters, by using the PNP equations. Thus the only role of the atomic detail (in predicting permeation of the type measured here) is to determine those few (average effective) parameters needed to predict *I-V* curves. We imagine that those few parameters (and the PNP equations) are sufficient because they more or less correctly describe shielding, and once shielding is more or less correctly described, the atomic details of structure (not involved in shielding) are not so important. This simplification is helpful because neither measurements of structure nor simulations of motion are likely to be possible on the time and length scale of permeation (~100 ns, 0.1 Å) for some years. We are certainly aware how fortunate we are: clearly this simplification does not apply to all properties of the channel and probably not to some characteristics of permeation, as well. Many properties should depend on the atomic details of channel structure.

The curve-fitting procedures used here to determine $P(x)$ are described in some detail by Chen et al. (1997). Briefly, a set of parameters is chosen as an initial guess, e.g., diffusion coefficients D_j for each ion and parameters P_k that describe the profile of permanent charge $P(x)$. Solving the PNP equations then predicts the current flow in a pair of solutions at a given membrane potential (and the profiles of potential and concentration through the channel as well). Using one pair of diffusion coefficients D_j and one profile of permanent charge $P(x)$ for the selectivity filter, the PNP model is solved for every potential and pair of concentrations at which current was measured. A minimization routine (Chen et al., 1997) is used repeatedly to modify (it is hoped, to improve) the initial guess of D_j and the parameters β_k of $P(x)$. The parameter estimates are modified (according to the scheme that is the essence of the nonlinear curve-fitting software) until the sum of squared residuals between predicted and measured currents cannot be improved further (or the parameter estimates no longer change appreciably). The resulting values are "best least-squares" estimators of the parameters in the PNP model and give the "best fit" of the theory to the data. These estimators are well determined by the data presented here if the permanent charge $P(x)$ of CRC is described by the simplest function, according to the tests of singular values and tests using constrained parameter values described in detail by Chen et al. (1997). The spatially uniform profile $P(x) = P_0 = -4.2$ M fits the data quite well (Fig. 1). Not surprisingly, more detailed descriptions of the profile $P(x)$ fit the data better, as we shall see, but it is not clear whether the improved fit is meaningful, given the likely presence of systematic error in theory and experiments. Nonner et al. (unpublished studies) investigated a number of families of functions that form a "complete basis" (i.e., that can sum to represent any reasonable function, the way sine waves do in a Fourier series), and sums of Bessel functions seemed best, at least for the present channel:

$$P(x) = \beta_1 + \beta_2 J_0(\pi x/d) + \beta_3 J_0(2\pi x/d) + \beta_4 J_0(3\pi x/d) \quad (1)$$

where x is the location along the channel of total length d, J_0 is a Bessel function of the first kind of order zero, and the β_k are parameters that characterize the (effectively one-dimensional) distribution of fixed charge in the channel and are determined by curve fitting. That is to say, fewer terms of this series of Bessel functions were needed to fit the data than of series of other functions. The best fit profiles $P(x)$ determined with Bessel functions and step functions were indistinguishable, when even eight step functions were

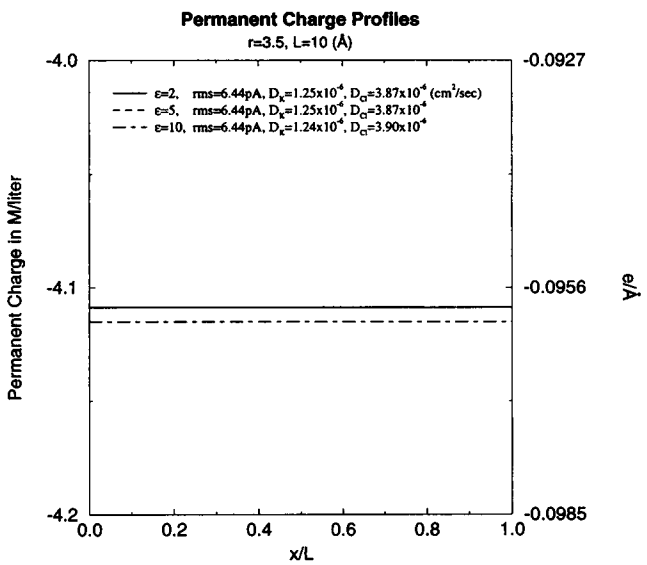


FIGURE 1 The permanent charge profile adopted as the best estimate of $P(x)$. The current-voltage relations measured in several solutions, fit with a uniform, spatially independent permanent charge, $P(x) = P_0 = -4.2$ M, diffusion coefficients $D_K = 1.3 \times 10^{-6}$ cm²/s, $D_{Cl} = 3.9 \times 10^{-6}$ cm²/s, and dielectric constants of 2, 5, and 10. Curves for $\epsilon = 2$ and $\epsilon = 5$ are indistinguishable. Singular values were {9016, 306, 14}. The theory fits quite well, with RMS error/RMS current = 6.4/67 summed over all solutions.

used, as is illustrated later, in Fig. 7. When these more detailed descriptions of $P(x)$ are used, the parameter values remain reasonably well determined, although some searching (by varying the initial guesses of parameter values) among local minima (in the value of the sum of squared residuals) was necessary to find the best fit.

Measurement of single-channel currents

Single-channel measurements were performed (Xu et al., 1996) between 23°C and 25°C by fusing proteoliposomes containing the purified cardiac muscle Ca^{2+} release channel with Mueller-Rudin-type bilayers containing phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in the ratio 5:3:2 (25 mg of total phospholipid per ml *n*-decane). The side of the bilayer to which the proteoliposomes were added was defined as the *cis* side. A strong dependence of single-channel activities on *cis* $[Ca^{2+}]$ was used to indicate that the large cytosolic ("foot") region of the channel faced the *cis* chamber. The potential on the *trans* side of the bilayer was defined as the ground (zero). Single-channel currents were recorded in 2 mM KHEPES, pH 7.5 buffer containing 4 μM Ca^{2+} and the [KCl] listed in Table 1. Data acquisition and analysis were performed with a commercially available software package (pClamp, Version 6.0.3; Axon Instruments).

RESULTS

The current-voltage relations of the open CRC channel were measured in the KCl solutions listed in Table 1 and are shown in Fig. 2, *A–D*. In both symmetrical and asymmetrical solutions, nearly linear *I-V* curves were obtained from −150 mV to +150 mV. Although linear *I-V* relations are hardly a surprise when the concentrations of permeant ions on both sides of the channel are the same, they are more of a surprise when the concentrations are very different. One might expect the current flow to rectify simply because the average concentration of ions in the channel presumably depends on whether ions flow from high to low or low to high concentrations, particularly if those concentrations differ by a factor of 10–20.

The lines in Fig. 2, *A–D*, are predicted by PNP equations when the channel has an effective fixed charge of $P(x) = P_0 = -4.2$ M and the diffusion coefficients are $D_K = 1.3 \times 10^{-6}$ cm^2/s and $D_{Cl} = 3.9 \times 10^{-6}$ cm^2/s. The concentration $P_0 = -4.2$ M is equivalent to a charge of $0.97e$ spread uniformly along a selectivity filter of 10 Å length, giving a linear charge density of $-0.097e$/Å. The fact that the estimated total charge is so close to an integer has not escaped our attention, although it has not captured it either. The estimate is probably a coincidence, but only structural information will tell for sure.

There is some significant misfit between theory and experiment in highly asymmetrical solutions, and at large potentials, but overall the fits are quite good: if concentrations are used to describe the solutions, as in the figures shown here, the fit is 6.4/67 (RMS residual/RMS current) from 314 measured currents in 12 solutions. If activities are used, the fit is 4.9/67. Because the estimates of the error of each measured *I-V* point are not available, the RMS residual is used instead of the χ^2 statistic.

The misfit was systematically investigated by fitting each *I-V* curve (from each pair of solutions) one at a time, allowing only one parameter to vary, keeping the other parameters at their mean values. For example, the data in the 250 || 50 mM solution were fit by varying only the permanent charge density while all other parameters were kept at their mean value. (The resulting fit was, of course, much improved.) The *I-V* data from this pair of solutions were then fit another time, by varying only the diffusion coefficient D_K. Repeating this process for all solutions (and for each of the three adjustable parameters) shows that either a mean change in permanent charge of 5.7%, or a mean change in D_K of 5.7%, or a mean change in D_{Cl} of 458% is needed to produce optimal fits to the *I-V* curves measured in each solution. The very large value for the required change in D_{Cl} reflects how badly that variable is determined by our data, theory, and analysis. This is hardly surprising, given how little current is carried by the coion Cl^- (in the conditions we study). The channel contains between 0.001 and 0.1 Cl^- (co)ions, depending on conditions, whereas it contains close to one K^+ (counter)ion, in accord with the (net) permanent charge of the channel protein of nearly $-1e$.

The singular values of the fit (Chen et al., 1997) were {9016, 306, 14}, suggesting that the values of two parameters (presumably, P_0 and D_K) are well determined. The correlation functions (Table 2, determined from 314 data points) show that the conductance is determined with equal weight by both the concentration of cations (counterions) in the channel's pore (which is determined almost entirely by the fixed charge $P_0 = -4.2$ M $\cong 1e$ in the 10-Å length of the selectivity filter) and by the diffusion coefficient of the counterion K^+. The conductance of a channel of uniform large charge should depend on the product of the concentration and mobility (i.e., diffusion coefficient) of the permeable ions (common sense, buttressed by the analytical results of Syganow and von Kitzing (1995) and Peskin (personal communication)). The concentration of permeable

TABLE 1 Solutions

	Concentration (mM)											
Cis	250	250	250	250	250	250	250	1000	1000	1000	1000	2000
Trans	250	2000	1000	500	100	50	25	1000	500	250	100	100

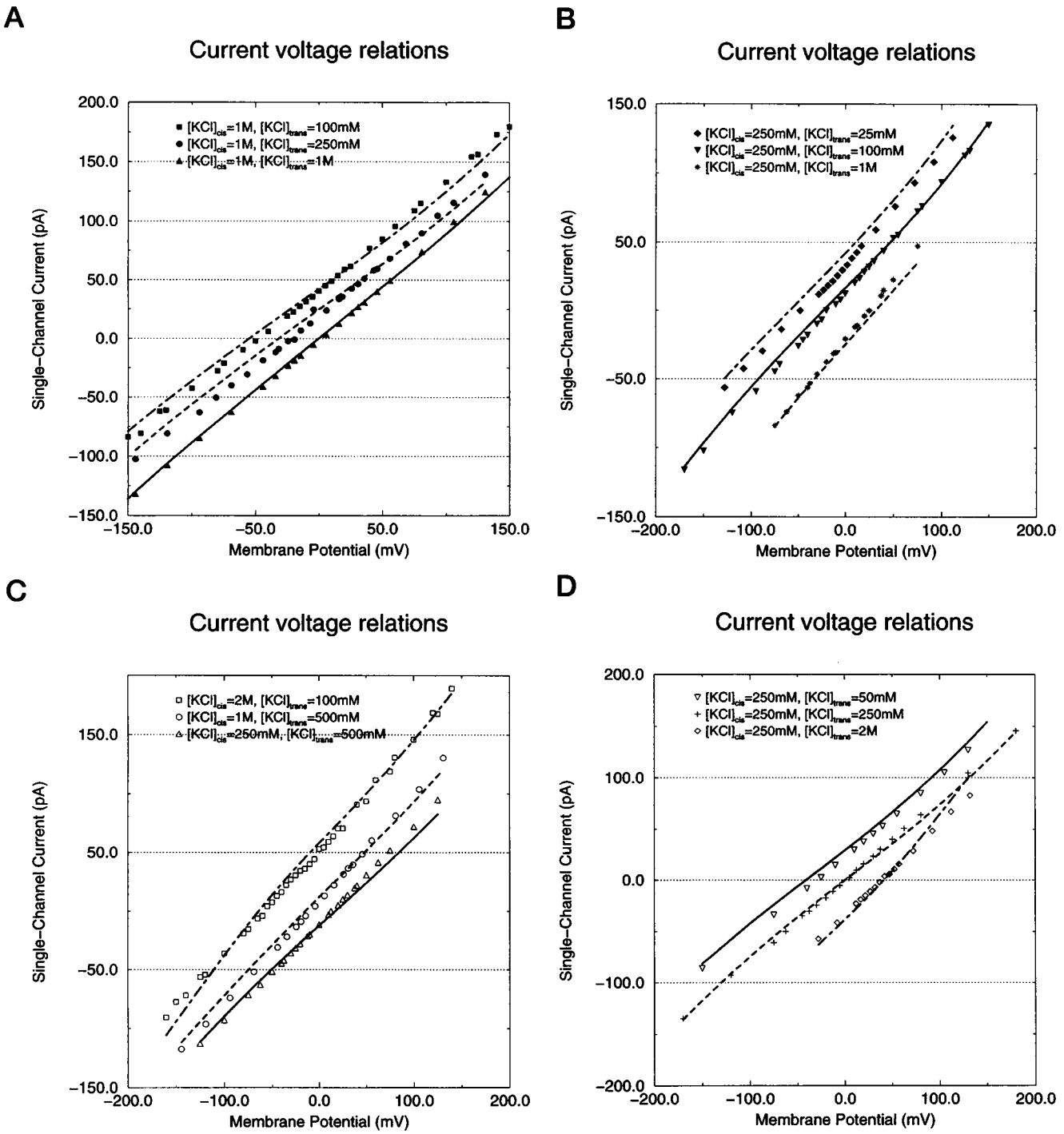


FIGURE 2 Current-voltage relations measured in the solutions indicated. The lines are the best fits of PNP with the parameters shown in Tables 2 or 4. The fits of the two models are not distinguishable by eye. Note that small (5.7%) changes in the value of the uniform fixed (i.e., permanent charge) could account for all of the deviations between theory and experiment (see text for details).

ions, in turn, is given to a good approximation by the concentration of permanent charge (because the system is approximately, but not exactly, electrically neutral, as in many ion exchange systems with a high density of fixed charge). Thus the effect of permanent charge and diffusion coefficient on the current is more or less the same, in a given pair of solutions and at a given transmembrane potential. It is not surprising then that estimates of these parameters are highly correlated (as in other barrier crossing problems; cf. Fleming and Hänggi, 1993).

On the other hand, the requirement that a single value of diffusion coefficient and a single value of charge density fit

TABLE 2 Parameter estimates: uniform permanent charge

Parameter estimate (±SD)	Correlation coefficient D_K	D_{Cl}	P_O
1.25×10^{-6} cm²/s (±0.13) = D_K	1	−0.88	−0.998
3.87×10^{-6} cm²/s (±0.44) = D_{Cl}	−0.88	1	−0.88
−4.17 M (±0.45) = P_0	−0.998	−0.88	1

A total of 314 data points from 12 solutions were used to estimate the parameters and correlation coefficients.

all of the data measured over a wide range of solutions and potentials allows the parameters to be separately estimated. At the reversal potentials (for example), the fixed charge density and the diffusion coefficients do not have the same effects. Thus measurements there, at that most traditional place, near the reversal potential, when the total current is nearly zero, allow separate identification of the parameters.

Curve fits with constrained parameter values and the analysis of singular values of the curve fits (both as described in Chen et al., 1997) show that our estimates of parameters are reliable, within reasonable bounds, despite the correlations reported. It also should be noted that estimates of changes of parameters between wild type and mutants of channels of known three-dimensional structure (Tang et al., 1997) are within 5% of the values known from their crystal structures.

In the calculations of this paper, the channel's filter (i.e., the narrow region that essentially determines open channel permeation) was chosen to have a length of 10 Å (Tu et al., 1994; Tinker and Williams, 1995). The channel diameter was chosen to be 7 Å because choline$^+$, Tris$^+$, and glucose can permeate (Meissner, 1986; Smith et al., 1988), whereas sucrose cannot (Meissner, unpublished studies). The dielectric constant (ϵ_p) of 5 was chosen as a reasonable number. The choice of the value has surprisingly little effect (for this particular profile of permanent charge), as shown in calculations that examined the fits for different values ranging from ϵ_p = 2–10, with a given profile of $P(x)$. The fits were not distinguishable and the estimates of parameter values were nearly the same (Fig. 1).

The atomic interpretation of the profile of charge $P(x)$ shown in Fig. 1 is of great interest and should be reevaluated once the structure of the selectivity filter is known and a more realistic theory is available. But some discussion is worthwhile, even with our present limited knowledge and theory (because our work shows that little atomic detail is needed to predict the properties of the open channel). Thus the substantial number of coordinates needed to specify the location and momenta of the atoms of CRC must "average out" to determine permeation; i.e., it must be somehow possible to reduce the coordinates of all the atoms of the protein to just the three (time-independent) numbers that determine permeation in the solutions shown in Table 1. This averaging is dramatic because of the size of the channel protein and because of the gap between the (shortest) time scale of atomic motion (say, 5×10^{-16} s) and the (shortest) time scale of measurements of permeation (say, 5×10^{-6} s). The number of coordinates needed to specify the location and momentum of the atoms of the channel is large, six times the number of atoms. The number of coordinates needed to specify how these fluctuate in time is much larger, because positions fluctuate significantly many times during the measurement of a single estimate of open-channel current; in fact, they fluctuate some $10^{10} = (5 \times 10^{-6})/(5 \times 10^{-16})$ times. The number of atoms necessary to determine the potential in a 1-cm^3 bilayer setup (if Coulomb's law is used instead of Poisson's equation) is very large (some 10^{20}). Thus direct computation of the current, by "adding up" successful trajectories, poses certain difficulties, given the finite word length and round-off error of numbers in computers. And direct computation of the potential seems impossible. Rather, a theory of the average current and average potential themselves will be needed to predict the biologically important properties of the open channel, namely, the effects of membrane potential and concentration on current flow. PNP is one such theory, perhaps the simplest.

What is surprising is that such an averaged description of the channel protein, using only three parameters (if one counts the diffusion constants as properties of the protein), can predict the averaged current, the I-V relations measured in such a range of conditions. Of course, the atomic details of the protein are important for other functions of the channel (e.g., gating) and for other characteristics of permeation as well (e.g., selectivity).

Although the insignificance of atomic detail surprised us, it did not surprise several of our collaborators who are professional mathematicians or chemists. The experimental data had a simple structure, and so they expected that a theory would involve only a few adjustable parameters, if the theory described the underlying physics more or less correctly. In this physical/mathematical approach, the channel should be described with no more detail than is needed to predict the data (that describe the phenotype of the system, its biologically interesting function).

The equations of the theory then transform the (sparse) atomic detail of the structure of the channel into predictions of its function, its I-V curves. They do this by predicting the profiles of ion concentration and potential along the channel—for every transmembrane potential and each pair of

TABLE 3 Dependence of rate constants on ion concentration and potential

Transmembrane potential V (mV)	K^+ rate constant k_f ($K+$)	K^+ rate constant k_b($K+$)	Cl^- rate constant k_f (Cl^-)	Cl^- rate constant k_b (Cl^-)
	μs^{-1}	μs^{-1}	μs^{-1}	μs^{-1}
KCl: 250‖250 mM				
0 mV	148	8.92	412	6838
100 mV	585	0.737	34.0	2.70×10^4
KCl: 250‖50 mM				
0 mV	59.7	0.722	828	6.85×10^4
100 mV	163	4.12×10^{-2}	47.4	1.87×10^5

TABLE 4 Parameter estimates: nonuniform permanent charge $P(x)$

Parameter estimate (±SD)	Correlation coefficient					
	D_K	D_{Cl}	β_1	β_2	β_3	β_4
1.48×10^{-6} cm²/s (±0.2) = D_K	1	−0.91	−0.87	−0.69	−0.01	0.29
4.12×10^{-6} cm²/s (±0.9) = D_{Cl}	−0.91	1	−0.94	0.86	0.097	−0.42
−4.82 M (±1.1) = β_1	−0.87	−0.94	1	−0.92	−0.31	0.62
8.12 M (±2.0) = β_2	−0.69	0.86	−0.92	1	0.15	−0.50
−4.09 M (±8.0) = β_3	−0.01	0.097	−0.31	0.15	1	−0.93
−9.95 M (±11.3) = β_4	0.29	−0.42	0.62	−0.50	−0.93	1

The permanent charge is $P(x) = \beta_1 + \beta_2 J_0(\pi x/d) + \beta_3 J_0(2\pi x/d) + \beta_4 J_0(3\pi x/d)$, where d is the length of the channel, and J_0 is a Bessel function of the zero order. A total of 314 data points from 12 solutions were used to estimate the parameters and correlation coefficients.

concentrations (equation 9 of Chen et al., 1997)—and from the profiles, the current flow (equation 11 of Chen et al., 1997).

Figs. 3 *A* and 4 *A* show the potential profiles predicted by the PNP equations in particular solutions. Figs. 3 *B* and 4 *B* show the concomitant profiles of concentration. Note that the horizontal axis has different (linear) scales inside the channel and in the baths to accommodate the different Debye lengths in the different regions.

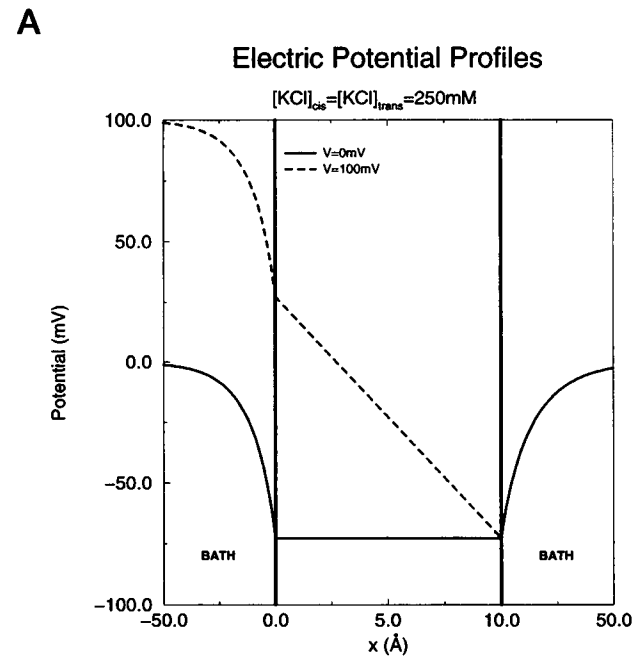
In the PNP equations, the parameters that determine the *I-V* relations also determine the profiles of potential and concentration. No other parameters enter at all. The shape and size of the profiles of concentration are different in different solutions—indeed, they are different at different transmembrane potentials—because the contents of the channel depend on the driving force (i.e., free energy difference) between the channel interior and both baths. If the contents of the channel vary, and the permanent charge density stays fixed, the total charge must vary, and so, on the most general of principles, the potential must vary. Thus the potential profile is different in different solutions and, indeed, at different transmembrane potentials. Flux and the rate constants that describe it tend to be an exponential function of transmembrane potential V and of the potential profile $\varphi(x)$, as can be seen by combining Eqs. 2 and 4 of the Appendix, and so the effects on current and rate constants are severalfold. A table of the forward and backward rate constants for K^+ and Cl^- are given in the legend of Fig. 4.

These conclusions depend on very general arguments and not on any details of our PNP equations. In fact, this argument is simply a fancy way of saying that shielding is a dominant determinant of the potential profile (and thus flux), a fact long accepted in the Debye-Hückel theory of ionic solutions, the Gouy-Chapman theory of interfaces, and the Poisson-Boltzmann theory of proteins, although not perhaps in enzymology (Hill, 1977, 1985; Walsh, 1979; Stryer, 1995) or channology (Hille, 1975, 1992).

The concentration profiles (Figs. 3 *B* and 4 *B*; note the logarithmic vertical axis) show that the selectivity filter of CRC is mainly occupied by K^+ ions, under the conditions shown, and the occupancy (by K^+) is much less dependent on transmembrane potential than is the profile of potential, according to PNP. The occupancy by K^+ (at zero and at ±100 mV membrane potential) ranged from 0.96 to 1.04, depending on the solutions. The profiles of Cl^- change much more than the profiles of K^+, ranging from 0.001 to 0.12 under the same conditions, but the change has a small effect on conductance, because the Cl^- concentrations are much smaller than the K^+ concentration. The concentration of K^+ ions is "buffered" by the high concentration of (mostly negative) permanent charge lining the pore of CRC, and it is this "buffering of counterions" that gives rise to the constant conductance (linear *I-V* curves) seen in our experiments. This buffering may also explain why PNP works as well as it does as a description of CRC, when similar theories do not predict the properties of bulk solutions very well: according to our analysis, the important region of CRC is well buffered from external disturbance by the high density of fixed charge lining the channel's pore, as long as the concentration of permeant is not too large. However, this buffering does not explain why PNP fits quite well *I-V* relations from channels with much lower densities (Chen et al., 1997) and more complex profiles of permanent charge (Chen et al., 1995b, 1997; Tang et al., 1997).

Previous work (Chen et al., 1997) predicts that changes in membrane potential and bath concentration produce large changes in the predicted potential profile in a synthetic channel with some −0.5 M permanent charge density spread along some 20-Å length. The filter of the CRC channel is more highly charged and seems shorter than that, as one might expect in a natural channel. The CRC filter seems to have a net permanent charge of some −4 M, corresponding to ~1 electron charge spread uniformly along its 10-Å length. Thus its potential profile is very sensitive indeed to the ionic atmosphere nearby, in the baths and the channel's pore.

Perhaps one sees here a hint of a biological principle governing the design of channels and the regulation of open-channel permeation. Perhaps the CRC channel is a rigid molecule designed to pack as high a density of fixed charge in as small a region as possible, producing as little friction as possible for the permeating ion. Perhaps the CRC channel is designed just so a simple mechanism like shielding will be the dominant determinant of its conductance. In this way, evolution could ensure a simple relation between the permanent charge (controlled by the genome) and the current through the channel (one of the genome's phenotypes). The design has the added advantage of being ho-



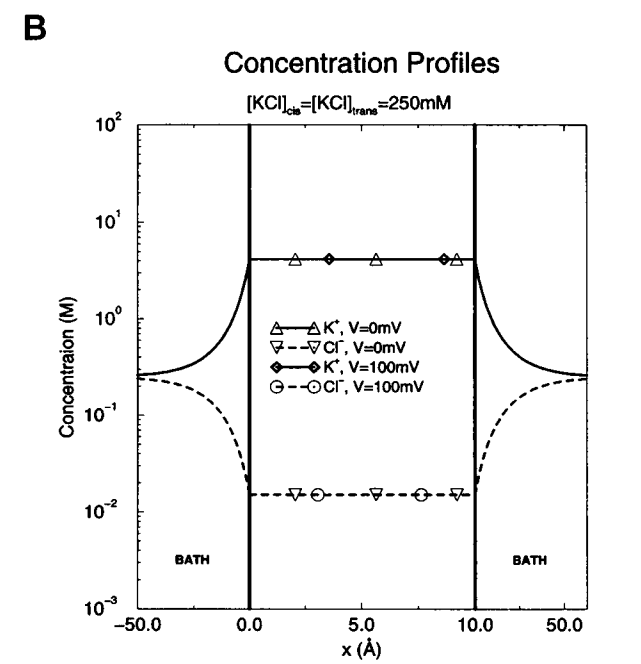
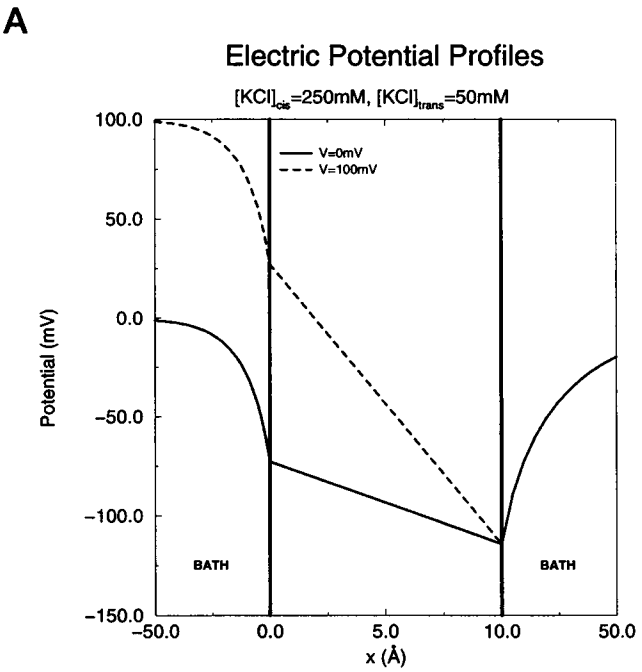


FIGURE 3 (*A*) Profile of the potential in symmetrical 250 mM solutions. Note the large region in which the potential in the baths differs from its bulk value (for this reason the scale of the horizontal axis is different inside the channel, $0 < x < 10$ Å, and outside the channel in the baths, $x < 0$ and $x > 10$ Å). This region is so large because the concentration of permanent charge at the ends of the channel is so large (see Fig. 1) compared to the concentration of the bathing solutions. The Debye length in the channel is thus much shorter than the Debye length in the bath. The potential profiles are clearly sensitive functions of transmembrane potential and bath concentration. See caption of Fig. 4. (*B*) Profile of the concentration of anions and cations in symmetrical 250 mM solutions with a logarithmic vertical axis and different horizontal scales inside and outside the channel. The concentration profiles show much less dependence on transmembrane potential and bath concentration than the potential profiles. This is to be expected from a channel like CRC with (nearly) linear *I-V* relations, and is not found in channels with more complex profiles of fixed charge and thus more complex *I-V* characteristics (Chen et al., 1995b, 1997; Tang et al., 1997).



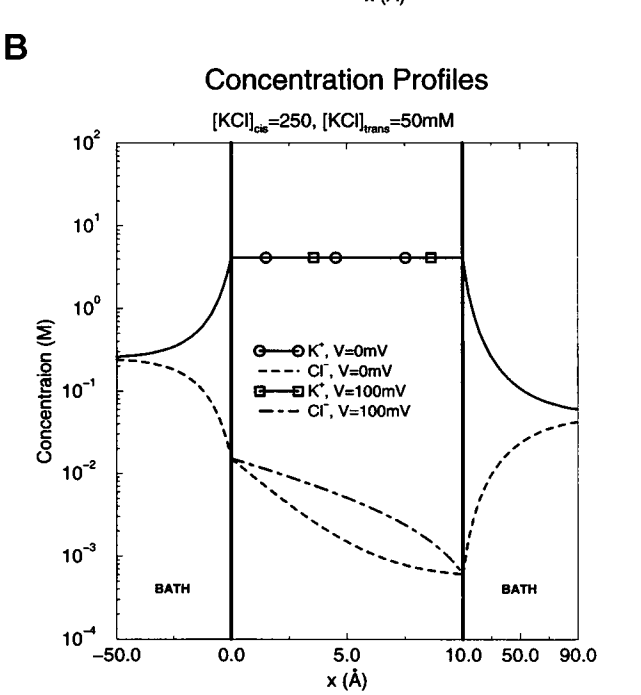


FIGURE 4 (*A*) Profile of the potential in asymmetrical 250 || 50 mM solutions. Note the large region in which the potential in the baths differs from its bulk value (for this reason the scale of the horizontal axis is different inside the channel, $0 < x < 10$ Å, and outside the channel in the baths, $x < 0$ and $x > 10$ Å). The potential profiles are clearly sensitive functions of transmembrane potential and bath concentration. (*B*) Profile of the concentration of anions and cations in asymmetrical 250 || 50 mM solutions with a logarithmic vertical axis and different horizontal scales inside and outside the channel. The forward and backward rate constants for the permeation of each ion are determined by the potential profiles as shown in the Appendix, Eqs. 2–4. The dependence is considerable, as documented in Table 3. Roughly speaking, in this channel, changing one solution from 250 mM to 50 mM changes the rate constants by a factor of 2–10; changing the transmembrane potential by 100 mV changes the forward and backward rate constants by a factor of 10.

meostatic in the concentration of permeating ions (but not homeostatic in the electrical potential, of course, presumably because physics makes that difficult, if not impossible); that is to say, the concentration of permeant is well buffered from external disturbance by the high density of fixed charge, as long as the concentration of permeant is not too large.

Fig. 5 shows the potential profile predicted within the selectivity filter of the CRC with asymmetrical 250 || 50 salt at a transmembrane potential of 100 mV if 1) there were no ions in the channel or bath (*solid line*), 2) if ions are present in the baths but not in the pore (*dashed line*); 3) if ions are permeable (*dot-dashed line*). The difference in the curves shows the potential change produced by ions in the channel's pore. Those ions shield the permanent charge of the channel and change the potential profile by several *kT/e*. The figure also shows that mobile ions significantly modify the shape of the potential profile and thus produce an additional change in ionic flux. (Comparing the solid line or dashed line with the dot-dashed line shows that even the sign of the curvature, and hence the net charge in the pore, is altered.)

Because no current flows when no ions are present, the difference in curves in Fig. 5 provides some estimate of the importance of nonequilibrium effects. When current flows, the potential profile is nearly linear (in this channel under these conditions); when current is not allowed to flow, the profile is nonlinear. The nonlinearity in potential is several *kT/e* and thus will change current flow more than several-fold, given the exponential relation of flux and potential. The deviation from equilibrium is not a small effect. Thus theories constrained to equilibrium, like Poisson-Boltzmann, are unlikely to successfully predict current flow through a channel. It seems unwise to analyze the electrostatics of a channel at equilibrium, or in a bath devoid of ions, as in most simulations of molecular dynamics, if the goal is to understand the phenotype and biological function of the open channel, namely, the flux of these ions.

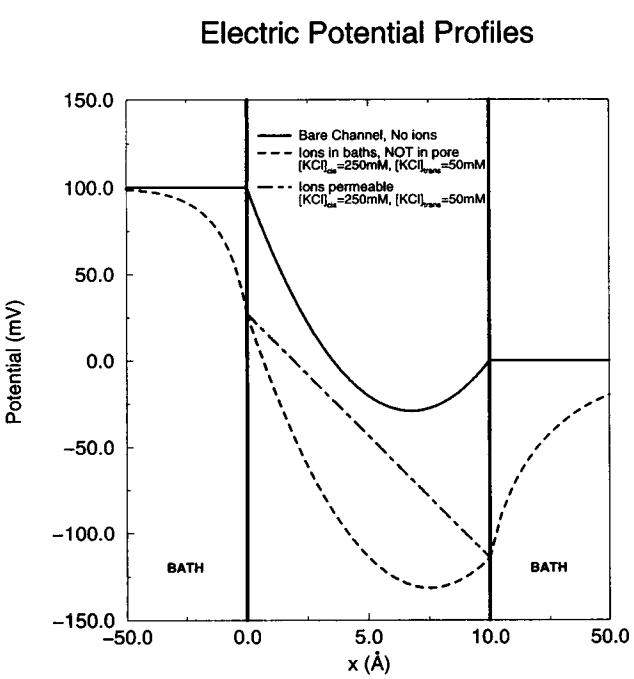


FIGURE 5 Potential profile of CRC with asymmetrical 250 || 50 salt if there were no ions in the channel or bath (——), if there were ions in the baths but not in the channel's pore (– – –), and if the ions were permeable (– · –). Note that potential profiles vary substantially with concentration, and so the rate constants given in Eq. 4 will also vary with concentration.

Fig. 6 provides a direct comparison of the best fits of PNP and a traditional four-barrier model using the parameters of cardiac CRCs reported in Tinker et al. (1992). The program implementing the barrier model was kindly provided by Osvaldo Alvarez (Alvarez et al., 1992). The prefactor *kT/h* was used, so our calculation overlaps those of earlier models. The fits by the barrier model were reasonable in symmetrical solutions but qualitatively unsatisfactory in the presence of asymmetrical salts. It may be possible to fit the data by a barrier model using a large number of parameters and *kT/h* as a prefactor; however, use of the standard prefactor of chemical physics (see Appendix) would prevent any theory with large barriers from fitting the data. Barrier theory, if used with the correct prefactor, cannot predict currents larger than a certain amount (corresponding to the smallest barrier that can still be called "large"). In the present case, barrier models cannot predict the observed currents, if the theories use the standard prefactor of chemical physics, and barriers are $3kT/e$ or larger. In fact, a barrier of $3kT/e$ predicts an open-channel conductance for CRCs of some 2 picosiemens, in 100 mM KCl, if the correct prefactor is used, compared to the hundreds of picosiemens we measure under those circumstances. On the other hand, PNP fits the conductance in this and all of the other solutions we have studied, using only a few parameters, that have reasonable values.

Not surprisingly, PNP fits the data better if more complex profiles of permanent charge are used. Two such profiles were determined (by curve fitting) by using the sum of four Bessel functions (Eq. 1) or an eight-step function. The complex profiles shown in Fig. 7 produce fits that are indistinguishable by eye from those shown in Fig. 2, *A–D*, but the statistics of the fits are improved significantly (RMS residual = 5.8 versus 6.4 with uniform P_0). The significance of this improvement over the result with a uniform profile of fixed charge is not clear, but the more complex profiles are shown here because preliminary measurements on the CRCs of skeletal muscle suggest that differences in the two channel isoforms may be resolvable in this way.

DISCUSSION

It seems clear that a simple theory of an open channel can predict the permeation of K^+ and Cl^- through the selectivity filter of the CRC channel in a wide variety of concentrations and a wide range of transmembrane potentials, if the theory computes the potential profile along the channel instead of assuming it.

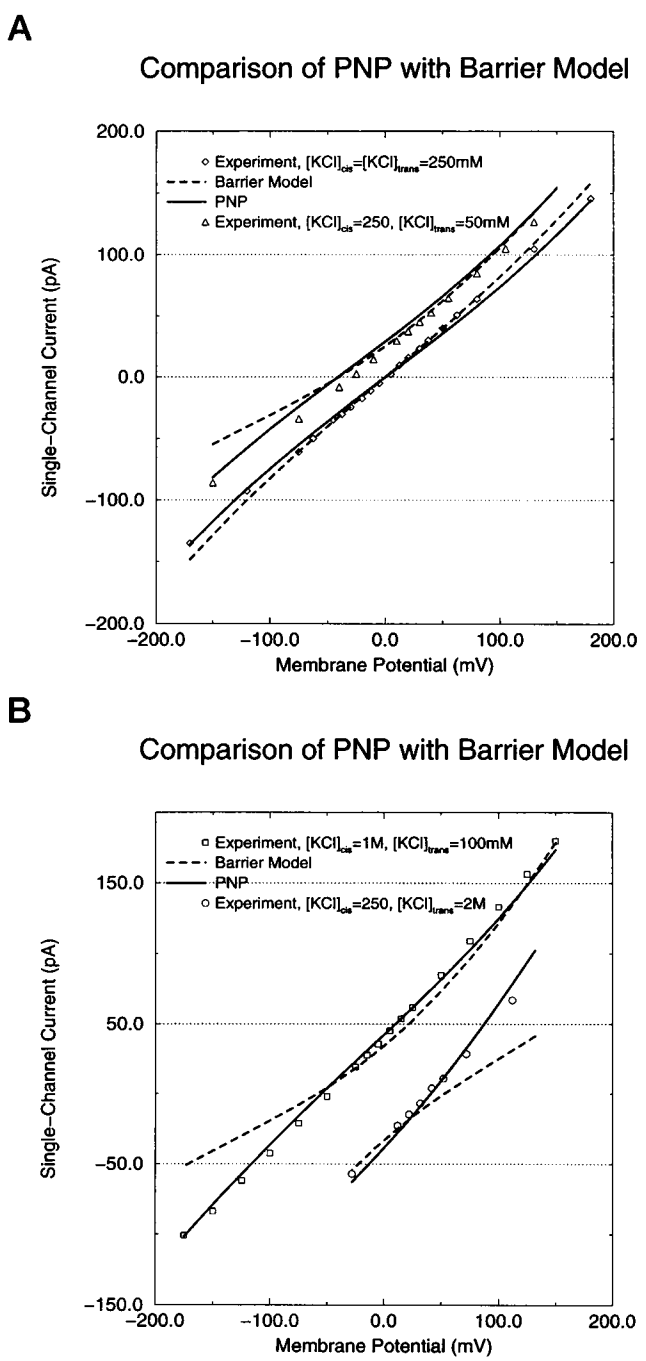


FIGURE 6 Comparison of PNP and barrier models in several solutions. Broken lines were obtained using the parameters given by Tinker et al. (1992). In particular, the prefactor kT/h was used, although Kramers' expression (see Appendix) would be a much better choice.

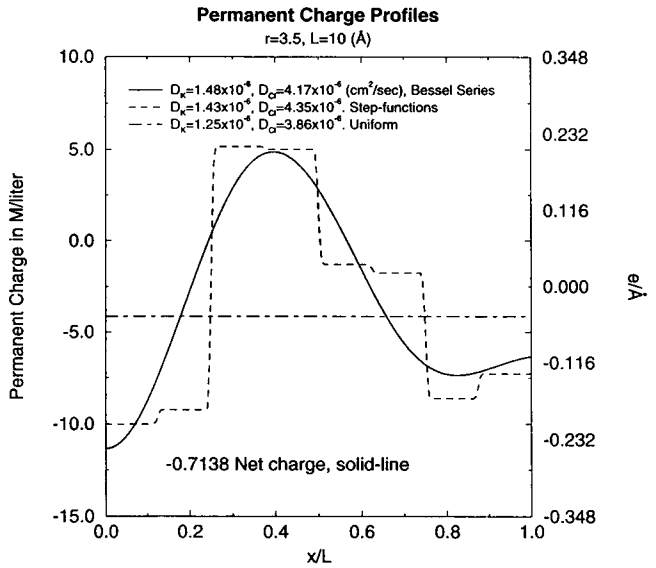


FIGURE 7 Two more complex profiles of permanent charge. $P(x) = -4.8 + 8.1J_0(\pi x/d) - 4.1J_0(2\pi x/d) - 9.9J_0(3\pi x/d)$ (in molarity, where x is the location in the channel of length d; J_0 is a Bessel function of order zero), and the diffusion constants in the pore are $D_K = 1.5 \times 10^{-6}$ cm^2/s, $D_{Cl} = 4.1 \times 10^{-6}$ cm^2/s. This profile produces somewhat better fits than a uniform profile. Its singular values are {7621, 281, 22.7, 15.7, 1.83, 0.426}, and so the parameters shown in Table 4 seem to be quite well determined. When an additional term $\beta_5 J_0(4\pi x/d)$ was added to improve the estimate of $P(x)$, the best least-squares value of its coefficient $\beta_5 = -0.52$ was much smaller than the other coefficients, the average magnitude of which was some 6.7 M. The SVD showed that β_5 was reasonably well determined: the singular values were {7645, 277, 22.6, 15.7, 1.97, 0.569, 0.222}, and so it seems that just the three Bessel functions (and constant) of this figure "exhaust the [information content of] the data." They describe the permanent charge as well as it can be described, given the limitations of theory and experiment. The data were also fit with a profile of permanent charge made of the sum of eight step functions. Note that it converges to give much the same estimate of diffusion coefficients and profile of permanent charge.

The theory does not fit perfectly, of course, particularly at large currents and in high salt concentrations; this might have many causes. Interestingly, if concentrations in the bath are replaced by activities, fit is significantly improved, particularly in the 2 M || 100 mM and 250 mM || 2 M solutions. The RMS residual for all solutions is then reduced to 4.9 pA. The original and residual misfits may come from ambiguities in the calculation and definition of activity in the bath: no one knows how to compute activity coefficients a priori in concentrated (i.e., physiological) ionic solutions (see the 18 references cited by Krukowski et al., 1995). When spatial gradients of activity are present (as in the bath and in the channel in the present situation), one faces a nonequilibrium problem. No one yet knows how to define variables analogous to free energy or activity away from equilibrium (Keizer, 1987; Lee and Rasaiah, 1994; Schönert, 1994; Vlad and Ross, 1995).

Misfits might also be produced by other phenomena not included in the theory, such as nonequilibrium effects, e.g., 1) water flow, which must be present in these asymmetrical solutions; 2) a drop in electrical or chemical potential produced by flux across an "access resistance" (see Chen and Eisenberg, 1993b); 3) heating of the solution, ions, and/or protein (Chen et al., 1995a); or by other effects, e.g., 4) a small change in structure (6% would do; see Results), which is not unlikely when ionic strength ranges from 20 mM to 2 M; or 5) by an effect of the electric field (really of the electrochemical potential) on the ionization state of acidic

or basic residues of the channel protein, i.e., an effect of large electrochemical potentials on the density of permanent charge (Warshel and Russell, 1984; Davis and McCammon, 1990; Honig and Nichols, 1995).

Nonetheless, the wonder is that PNP fits such a wide range of data so well. Evidently, water flow, access resistance, and changes in ionization or conformation (of more than 6%!) are not too important, at least for this channel under these conditions.

We suspect that PNP fits this well because it is a self-consistent theory that computes the potential profile from all of the charges present. PNP is the first and perhaps simplest theory of this type, but it has obvious deficiencies (Eisenberg, 1996): it neglects atomic detail and short-range electrostatic forces that are likely to be important to some channel functions, e.g., selectivity between ions. It ignores the entry and exit processes where an ion dehydrates and resolvates. It treats single filing only as a consequence of average (i.e., continuum) electrostatic repulsion, and it does not include additional effects of the repulsion between individual ions (Bek and Jakobsson, 1994), although those additional discrete effects may not be very important when permanent charge is large enough to buffer and thus submerge other phenomena, like the effects of discreteness of charge. It does not treat unidirectional fluxes individually, but lumps them together into a net flux (thereby removing any terms common to both the unidirectional influx and efflux), and so PNP (in its present form) cannot be used to predict the ratios of unidirectional fluxes characteristic of single-file systems (Eisenberg et al., personal communication). Each of these deficiencies must be addressed. We (and others) are trying. In particular, a preliminary version of PNP that includes single filing, using the method of Barkai et al. (1996), has been developed by Schuss (Schuss et al., personal communication).

In the meantime, however, the success of PNP in fitting such a wide range of currents, with qualitatively different dependence on transmembrane potential, in so many solutions, and in some five channel types (Chen et al., 1995b, 1997; Tang et al., 1997), suggests that the theory should be used as a guide and target for experimentation on the open channel, instead of barrier models (see Appendix) and traditional diffusion models (e.g., constant field).

Such experimentation will show where the evident theoretical deficiencies of PNP limit its practical utility. Experiments with porin (a channel of known structure) and mutants (with known changes in permanent charge) will cast light on the meaning of the effective parameters of the theory. Preliminary work (Tang et al., 1997) suggests that changes in the parameters are reliably estimated by curve fitting with PNP, showing that the change in permanent charge is not an effective but an actual parameter, at least for that channel under those conditions. Measurements with monovalent ions other than K^+ will show if the diffusion coefficients estimated with PNP behave as they do in free solution. Measurements with mixtures of monovalents will show if PNP can describe selectivity among these ions. Clearly a theory that treats occupancy as cavalierly as PNP should fail when describing permeation in (a range of) mixed solutions. But it is important to see where, how, and why PNP does fail, so that the appropriate improvements can be made without making the theory too complex to be of practical use in fitting the large data sets of experimental results (from many pairs of solutions).

Similarly, measurements should be made in divalent solutions, and in mixtures of mono- and divalent ions. Divalents are of particular interest in the cardiac CRC channel, because the channel exists to transport Ca^{2+}. Ca^{2+} flux is its phenotype. Theories of divalents in bulk solution do not do well in a wide range of concentrations, if at all in physiological concentrations or in mixed solutions. (This literature, which is particularly relevant for CRCs, can be found through the classical papers of bioelectrostatics (McLaughlin et al., 1981; McLaughlin, 1989) and in the recent chemistry literature (Kjellander and Mitchell, 1994; Booth et al., 1995; Ennis et al., 1995; Kjellander, 1995; Hummer et al., 1996; Kalko et al., 1996; Mehler, 1996).) Nonetheless, PNP should be tried even in these cases, to see where and how it fails, to focus theoretical attention on the practical issues that demand resolution. Logically, this work would be done on channels with known structure, that allow calcium permeation (e.g., the porins), but interest and support for work on CRCs is understandably high, and so perhaps work will not proceed in logical order.

Despite (or because of) its simplicity and success in fitting data, PNP is a frustrating theory, particularly when shielding effects are large, because they are so nonlinear and hard to predict a priori without actual numerical calculation. The shielding effects we find in CRCs are surprisingly large because the channel filter is (apparently) so short and narrow (~10 by 7 Å) (see Results for references), and its wall is so highly charged (~4 M, corresponding to $\sim 1e$ in the channel filter, according to the results presented here). The potential profile within a conducting channel is thus very different (e.g., often by kT/e at many locations) from what it would be in an empty channel, a hypothetical channel without ions to shield the permanent charge of the protein, or from what it would be in a filled channel at equilibrium that did not conduct current.

The nonlinear equations of PNP are straightforward—nearly trivial—to compute, once one knows how, although (re)inventing how (Chen et al., 1992; Chen and Eisenberg, 1993a; Chen et al., 1997; compare with the semiconductor literature cited in Jerome, 1995, and Lundstrom, 1992) and learning why (Jerome, 1995) were not at all straightforward. But after-the-fact calculations do not permit the understanding all scientists seek, before experiments or computations are done. After a computation is done, the profiles of concentration and potential along the channel (that are outputs of the theory) clearly explain why currents vary the way they do, as permanent charge, transmembrane potential, or concentrations are changed. But before the computation, those profiles, particularly the most important, the profile of potential, are hard to predict.

Study of the *I-V* relations predicted by PNP in many solutions might help in developing insight into its qualitative behavior, and many such plots have been made (mostly by our collaborator and friend Wolfgang Nonner, who has generously shared them with us). But "the goal of computing is insight, not numbers," and that goal has not yet been reached by extensive computation of PNP.

More promising is the analytical path. Barcilon et al. (1997) and Charles Peskin (personal communication) have used singular perturbation theory (cf. Kevorkian and Cole, 1996) to determine general qualitative properties of the PNP equations. Peskin has made particular progress by exploiting the large value of the permanent charge (in his *Lecture Notes on Neurobiology*). Peskin et al., (unpublished studies) are working on a related analysis of PNP itself, which should yield insight into CRC behavior, because it can be reasonably described by a uniform large $P(x) = P_0$.

Clearly, much more work is needed to test and then (it is hoped) exploit the PNP model of CRC. Measurements should be made in a wide variety of monovalent ions, to see if selectivity (of this type) is reasonably described. Modifications of the protein should be made (particularly of the permanent charge), and the effects on *I-V* curves predicted and measured. Most importantly, the theory and experiments should be extended to include the divalent ions of greatest functional interest in this channel.

APPENDIX: RATE CONSTANTS IN CHANNOLOGY

Rate models are used so widely in channology (Hille, 1975; Hille and Schwartz, 1978; Eisenman and Horn, 1983; Läuger, 1991; Andersen and Koeppe, 1992; Hille, 1992, are modern references) that we think it necessary to show explicitly how they arise in a diffusion theory applied to open channels.

Rate models of channels grew from the rate theory (sometimes called "transition state theory") of chemical reactions developed in quantum chemistry in the 1930s. Despite its popularity then, chemists realized that rate theory must be derived (Laidler and King, 1983). It is not a fundamental physical law of either quantum or statistical mechanics, and its use must be justified by derivation, simulation, and experimentation.

Rate theory was derived in two different traditions: those of equilibrium statistical mechanics (Johnson et al., 1974; Hille, 1975; Pechukas, 1976; Chandler, 1978; Hille and Schwartz, 1978; Eisenman and Horn, 1983; Levine and Bernstein, 1987; Steinfeld et al., 1989; Läuger, 1991; Andersen and Koeppe, 1992; Hille, 1992) and diffusion theory (evidently started by Kramers, 1940; see the definitive review of Hänggi et al., 1990, citing some 700 other references; also see the textbook presentations of Berry et al., 1980, and Robinson and Holbrook, 1972, and the recent book by Fleming and Hänggi, 1993, which contains a number of articles joining the two traditions).

The tradition of statistical mechanics has difficulty accommodating flux, because flux of all types vanishes at equilibrium, where statistical mechanics is derived. Thus phenomena that occur only when macroscopic flux flows (e.g., friction or frictional heating) are not natural components of theories in statistical mechanics.

The tradition of diffusion theory has difficulty accommodating atomic detail. Frictional phenomena are natural parts of diffusion theories, but the equations of molecular dynamics used to describe molecular motion in atomic detail do not include diffusion coefficients or explicit treatments of friction.

Statistical mechanics and diffusion theory must both be extended if their relationship is to be understood. Equilibrium ideas (like free energy and its components, energy and entropy) and atomic resolution must be present in the (extended) diffusion theories; and nonequilibrium ideas, like friction, must be present in the (extended) equilibrium theories.

The diffusion theory of channels started historically with the Nernst-Planck equations, the diffusion equations describing the concentration of charged particles, each of which following the random trajectory necessarily produced by friction (Goldman, 1943; Hodgkin and Katz, 1949; Hall et al., 1973; Levitt, 1982, 1984, 1985, 1986, 1987). Kim Cooper, then a graduate student of the biophysicist Eric Jakobsson and physical chemist Peter Wolynes, was (as far as we know) the first to use Langevin equations to describe the random trajectory of ions in a channel (Cooper et al., 1985, 1988a,b). We (and others) followed his lead. Eisenberg et al. (1995) provided a stochastic derivation of the Nernst-Planck equations, showing how those equations describe the probability density function for the location of an ion moving in a random trajectory. The stochastic derivation rationalized the analysis and demonstrated the generality of the simulations of Barcilon et al. (1993).

The stochastic derivation provides a pleasingly intuitive result. The flux of trajectories (and ions) is the sum of two unidirectional fluxes, each the product of a "source" concentration, "diffusion velocity" (D_j/d) and the appropriate conditional probability:

$$J_j = d[k_f C_j(L) - k_b C_j(R)] \tag{2}$$

$$\equiv \overbrace{\underbrace{C_j(L)}_{\substack{\text{Source}\\ \text{Concentration}}} \underbrace{\left(\frac{D_j}{d}\right)}_{\substack{\text{Diffusion}\\ \text{Velocity}}} \underbrace{\text{Prob}\{R|L\}}_{\substack{\text{Conditional}\\ \text{Probability}}}}^{\text{Unidirectional Efflux}} - \overbrace{C_j(R)\left(\frac{D_j}{d}\right)\text{Prob}\{L|R\}}^{\text{Unidirectional Infflux}}$$

Note that the total flux cannot itself be described (in any natural way) by a (single unconditional) probability, nor can the mean first passage time or contents of an ion in a channel. All of these quantities must be replaced by the appropriate (pairs of) conditional quantities because a number of the unconditional quantities are infinite in perfectly finite and well-posed situations, as found by Barcilon et al. (1993) and Eisenberg et al. (1995).

When the unconditional quantities are replaced by the appropriate conditional quantities, the flux through the channel can be described in a simple manner, e.g., as a unimolecular chemical reaction (Robinson and Holbrook, 1972):

$$L \underset{k_b}{\overset{k_f}{\rightleftharpoons}} R; \tag{3}$$

$$\text{where } k_f \equiv \frac{D_j}{d^2}\text{Prob}\{R|L\}; \quad \text{and } k_b \equiv \frac{D_j}{d^2}\text{Prob}\{L|R\}$$

In words: each flux can be described as a (unidirectional) chemical reaction without approximation, for any potential barrier $\Phi(x)$ with rate constants k_f and k_b (units: s^{-1}), determined by the conditional probabilities and diffusion velocities shown in Eq. 2, when concentration boundary conditions are in force that describe mathematically the constant-concentration/constant-potential conditions of a voltage-clamp experiment.

The conditional probabilities of Eqs. 2 and 3 require precise definition, including two boundary conditions that doubly condition the underlying trajectories, which must be described by the full (not reduced) Langevin equation, to allow the double conditioning. It was the assignment of these trajectories and boundary conditions that allowed Eisenberg et al. (1995) to specify and solve this problem, using the techniques of Schuss (1980) and Naeh et al. (1990).

The conditional probabilities of Eqs. 2 and 3 can be determined entirely numerically, by computing a random walk, or by simulating a full or reduced Langevin equation. All three numerical calculations are shown by Barcilon et al. (1993) (e.g., Figs. 4 and 5) (see also Cooper et al., 1985; Chiu and Jakobsson, 1989; Eisenberg et al., 1995). The conditional probabilities might also be determined from the simulations in atomic detail of

molecular dynamics (McCammon and Harvey, 1987; Brooks et al., 1988; Haile, 1992) or by using the Onsager-Machlup action formulation of Newton's laws, in the presence of thermal agitation (Onsager and Machlup, 1953; see modern application: Elber, 1996). The simulations fortunately require much less time than the derivation (Eisenberg et al., 1995) of their boundary conditions, which took many of us years of work (Cooper et al., 1985, 1988a,b; Chiu and Jakobsson, 1989; Barcilon et al., 1993).

Equations 2 and 3 are derived by using stochastic identities that merely assume the existence of conditional probabilities of location and so are true for a wide range of stochastic trajectories. Thus the derivation establishes the chemical reaction as a model of the open channel, and the meaning of the Nernst-Planck equations mathematically, without physical argument beyond that used in deriving the model of the open channel in the first place.

In the general case we can conclude, then, that the chemical reaction and Nernst-Planck equations are not a (perhaps vaguely derived) continuum approximation, but rather are an exact representation and description, even in atomic detail, if they use the conditional probability density functions of the location of discrete particles, as defined above and in the cited references. (See above. The meaning of the average potential profile $\varphi(x)$ of the Nernst-Planck equations is more subtle, if not problematic, and is discussed at length in Eisenberg (1996).)

In a special case, when friction is large (as in channels on the biological time scale) and well behaved (characterized by a single number D_j for each ionic species j), the statistics of the conditional trajectories (e.g., mean flux, first passage times, and channel contents of left and right trajectories) can be determined analytically (Eisenberg et al., 1995), using mathematical techniques developed by Schuss. In that special case, the conditional probability and rate constant can be written as

$$\frac{D_j}{d^2}\,\mathrm{Prob}\{R|L\} \equiv k_f = \frac{D_j}{d^2}\cdot\frac{\exp(z_jV)}{1/d\int_0^d \exp[z_j\varphi(x)/kT]dx} \qquad (4)$$

The normalized transmembrane potential V is defined as $V \equiv eV_{appl}/kT$.

If the potential profile $\Phi(x)$ is dominated by a large barrier, and satisfies certain other criteria, expressions for rate constants reduce to exponential expressions (Section viii of Barcilon et al., 1993; and eq. 8.4 and 8.5 of Eisenberg et al., 1995) reminiscent of rate expressions of reaction rate theory used widely in channology (Hille, 1975; Hille and Schwartz, 1978; Eisenman and Horn, 1983; Läuger, 1991; Hille, 1992). However, the prefactor of (the exponential expression derived from) diffusion theory is physically very different because it depends explicitly on friction, as noted by many biophysicists (see Cooper et al., 1985, 1988a,b; Chiu and Jakobsson, 1989; Läuger, 1991; Roux and Karplus, 1991; Andersen and Koeppe, 1992; Barcilon et al., 1993; Crouzy et al., 1994; Eisenberg et al., 1995) and even more physical chemists (Hänggi et al., 1990). For example,

$$k_f \xrightarrow[\text{barrier}]{\text{high}} \underbrace{\frac{D_j}{d\sqrt{2\pi}}\sqrt{|z_j\,\Phi''(x_{max})|}}_{\text{PREFACTOR}}\exp[z_jV - z_j\,\Phi_{max}(x_{max})] \qquad (5)$$

There is no controversy in the chemical literature about this expression or its prefactor. Exactly this expression is widely used there to describe the flux over high barriers. (The large barrier result is derived in the equilibrium tradition in Robinson and Holbrook (1972); Johnson et al., (1974); Pechukas (1976); Berry et al. (1980); Levine and Bernstein (1987); Steinfeld et al. (1989). The prefactor in those expressions is not simply kT/h; it includes a ratio of (factors of the grand) partition functions as well, and is in agreement with much experimental data. Fleming et al. (1986) and Schroeder and Troe (1993) both present and cite the large experimental literature.

The large barrier result is derived in the diffusion tradition by Kramers (1940), Gardiner (1985), Hynes (1985, 1986), Berne et al. (1988), Hänggi et al. (1990), and Fleming and Hänggi (1993). Many derivations (in both the equilibrium and diffusion traditions) are given by Hänggi et al. (1990), as are a detailed discussion of the prefactor and numerous (~700) references to the historical and modern literature. Fleming and Hänggi (1993) describe the current state of knowledge: they include articles describing experimental measurement of the prefactor, a succinct reconciliation of equilibrium and rate constant traditions using variational theory, and a powerful description of the limitations of any one-dimensional theory, along with other useful articles.

It is important to note that many modern books and reviews on transport (McQuarrie, 1976; Friedman, 1985; Ma, 1985; Chandler, 1987; Mason and McDaniel, 1988; Smith and Jensen, 1989; Spohn, 1991; Balian, 1992; Mahan, 1993; Bird, 1994; Cercignani et al., 1994; Garrod, 1995) hardly mention barrier or rate models at all, preferring to deal with the general situation, in which barriers can have any shape or size, which some channologists prefer (Hall et al., 1973; Schuss, 1980; Levitt, 1982, 1984, 1985, 1986, 1987; Cooper et al., 1985, 1988a,b; Chiu and Jakobsson, 1989; Barcilon et al., 1993; Eisenberg et al., 1995; Bek and Jakobsson, 1994).)

At first glance, the typical system of the chemical literature seems quite different from a channel. In most chemical experiments involving flux over high barriers, concentrations change as the flux flows, in contrast to most channel experiments in which concentrations (and potentials) are kept constant (as flux flows) by the active intervention of experimental equipment (i.e., by stirring or perfusion and by the voltage/patch-clamp amplifier). However, in one special case—when barriers are high enough—these different experimental conditions produce similar fluxes (Barcilon et al., 1993; Eisenberg et al., 1995): high enough barriers are rate-limiting in both cases, even though experimental conditions are different, as are the boundary conditions that describe them mathematically. When barriers are high enough, the chemical and channel systems are nearly the same, probably because in that special case the system is nearly at equilibrium and experimental and boundary conditions do not matter very much.

The numerical value of the prefactor of Eq. 5 can be estimated easily if the potential profile $\Phi(x)$ is a symmetrical parabolic barrier spanning the whole length d of the channel, with maximum size $\Phi_{max}(x_{max})$, much larger than the applied (i.e., transmembrane) potential V. Then, for example,

$$k_f \xrightarrow[\text{high barrier}]{\text{PARABOLIC}} \underbrace{\frac{2D_j}{d^2\sqrt{\pi}}\sqrt{|z_je\varphi_{max}(x_{max})/kT|}}_{\text{PREFACTOR}}\exp[-z_je\varphi_{max}(x_{max})/kT] \qquad (6)$$

where we use the dimensional potential $\varphi(x) = \Phi(x)kT/e$ and $x_{max} = d/2$. The diffusion (i.e., Kramers) prefactor depends on the diffusion coefficient and channel length, which do not appear in the hopping prefactor kT/h at all. The diffusion prefactor varies inversely with the (square root of the) temperature, whereas the hopping prefactor depends linearly on temperature. The Kramers prefactor depends on the type of permeating particle; the hopping prefactor is independent of the type of particle(!). These different properties have made it easy for chemists to determine the prefactor that actually describes the properties of solutions and condensed phases (Fleming et al., 1986; Schroeder and Troe, 1993).

Now if the barrier is, say, $4kT/e$ high and 1 nm long and the diffusion coefficient is some 1.3×10^{-6} cm^2/s—as we find for K^+ in the "filter" of the CRC channel, which is not dissimilar to the values others find for other channels (Dani and Levitt, 1981; Chen et al., 1995b, 1997; Tang et al., 1997)—the numerical value of the (diffusion expression for the) prefactor (for K^+) is $\sim 2.8 \times 10^8$ s^{-1}. The numerical value of the usual prefactor in the hopping theory is kT/h, which is $\sim 2.2 \times 10^4$ times larger, $\sim 6.3 \times 10^{12}$ s^{-1} at biological temperatures. As one might expect, ions hopping over barriers experience much less friction than ions diffusing over them, and the amount of friction will depend on the identity of the ion.

The effect of friction (i.e., the ratio of the two expressions for the prefactor, one general, the other for K^+) is numerically equivalent to a change in the potential barrier of $\ln(2.2 \times 10^4) \cong 10kT/e$. For example, a barrier of height $13kT/e$, analyzed with the kT/h prefactor, produces the same rate constant as a barrier of height $3kT/e$, analyzed with the Kramers prefactor. Or, in a more ominous example, a barrier of $10kT/e$—which is more than large enough to be described by the high barrier approximation in Eq. 5 or 6—becomes 0 kT/e, which cannot be described by a high

barrier approximation, because it is no barrier at all. Indeed, almost all barrier models of open channels use kT/h as a prefactor and postulate barriers in the range of $3kT/e$ to $12kT/e$; for example, the barrier heights used to model CRC are $5.5kT/e$ (table 1 in Tinker et al., 1992, p. 498). Barrier models with such barriers cannot come close to fitting the open-channel current found in most channels (Conley, 1996a,b, 1997) if the correct prefactor is used.

It is evidently quite important to settle on the correct value of the prefactor for channel permeation before a high barrier approximation is used. The channel length d is unlikely to be short enough; the diffusion coefficient D_j to be large enough, or the same for different ions; or the potential barrier $|\varphi_{max}|$ to be large enough to allow kT/h to approximate the diffusive prefactor:

$$\frac{2D_j}{d^2\sqrt{\pi}}\sqrt{|z_j e\varphi_{max}(x_{max})/kT|}$$

Of course, even if the numerical values were not too different, the meaning of the prefactors would be very different, because their temperature dependence is so different, and one depends on friction and the height of the potential barrier, the identity of the permeating ion, and the length of the channel, whereas the other looks more like a "constant of nature," independent as it is of the properties of the channel and ion.

Careful reading of the classical theories of barrier crossing in channology (Hille, 1975, 1992; Hille and Schwartz, 1978) shows us how to reconcile the two treatments. Those theories have defined a barrier height by its free energy (temperature times entropy plus electrical energy), not its (electrical) energy. In that case, the two treatments and prefactors can be reconciled if the frictional prefactor of diffusion theory is equated to the "activation entropy" of rate theory.

Unfortunately, the "activation entropy" is not likely to be small or have a small effect, or be the same under all conditions of biological and experimental interest, because the trajectories of the ion (that determine the entropy) are quite different qualitatively and quantitatively in the bath and in the channel. The motion is three-dimensional in the bath but (nearly) one-dimensional in the channel; and the diffusion coefficient of ions in the bath is generally much higher than in the channel's pore (Dani and Levitt, 1981).

The activation entropy of traditional barrier theories is more vaguely defined than the prefactor of diffusion theory, until the dependence of activation entropy on temperature, friction, and channel length is determined. This dependence is not derived or displayed in traditional theories of barrier crossing or in most barrier models, either, and so data measured with different permeating ions (and thus, most likely, unequal diffusion coefficients), at different temperatures, or in systems with unequal channel lengths, cannot be compared by using the "activation entropy" or "activation free energy" (Hille, 1975, 1992; Hille and Schwartz, 1978) formulation. Measurements of the value and functional dependence of the prefactor (on temperature, diffusion constant, etc.) are available in the chemical literature (e.g., Fleming et al., 1986; Hänggi et al., 1990; Fleming and Hänggi, 1993; Schroeder and Troe, 1993). They are incompatible with the expression kT/h, and in fact are close to the Kramers expression (Eq. 5) or its generalizations, under a wide range of conditions in many systems.

"Barrier heights" determined experimentally in channology (using rate theory with the kT/h prefactor) represent the free energy barrier to ion translocation. Free energies are, of course, a perfectly adequate representation of barrier heights (if barriers in open channels are in fact high), as long as the free energy is not confused with the potential energy: free energy includes entropy, and the entropy term changes current by a factor of $\sim 2 \times 10^4$, as we have seen. Thus a verbal model or mathematical theory (or simulation of molecular dynamics) must compute the entropy as well as the energy if it is to be compared with experimental estimates of barrier heights.

If a theory calculates just the barrier of potential energy—using Coulomb's law or Poisson's equation or a verbal version of either, to describe binding at a charged site, for example—it must not ignore the difference between potential energy and free energy, it must not ignore the entropy component of free energy, and it must not use kT/h as the prefactor, or large errors ($\sim 2.2 \times 10^4$) will occur in predictions of the current or estimates of barrier height ($\sim 10\ kT/e$). In particular, molecular models of binding sites, whether verbal or quantitative, must explicitly estimate both the energy and entropy terms if serious quantitative errors are to be avoided, as we have seen.

If a barrier model ignores the dependence of the entropy term on the type of permeating ion, or if it ignores the dependence on the diffusion coefficient, temperature, barrier height, and channel length, serious qualitative errors are likely to occur as well. In particular, traditional barrier models are likely to give qualitatively misleading results (because they use kT/h as a prefactor) if they are used to compare experiments involving different ions (with different diffusion coefficients and thus different prefactors and activation entropies), experiments with mutated or modified channels (which have modified potential barriers and thus modified prefactors and activation entropies), or experiments with different concentrations of ions (which are likely to have different potential barriers (Eisenberg, 1996) and thus different prefactors and activation entropies).

We have seen that the general expressions, Eqs. 2–4, determine the flux and (and its rate constant) exactly, for small as well as large barriers, without concern about prefactors. The general expressions have unambiguous meaning, and their functional dependence is widely accepted in the chemical literature. They are simple to compute, using generally available software that takes virtually no time to execute. Presumably for these reasons, a number of chemists do not use the high barrier theories at all (citations above). Perhaps channologists should follow this practice, at least when studying open channels.

It seems worthwhile to list the difficulties (documented in this Appendix and the Results) facing traditional barrier models of open channels, so that scientists can be aware of what they are assuming when they use them:

1. Barrier models of channels are based on a view of the trajectories of ionic motion in condensed phases which has been shown to be false, both experimentally and theoretically. Ions do not hop as they move in such systems; rather, they follow diffusive, nearly fractal paths.

2. Barrier models of channels assume potential barriers that are independent of the concentration of ions in the baths and of transmembrane potential. That is to say, they ignore the effects of the charged contents of the channel (and other mobile charges) on the potential barrier. These effects are large; indeed, these effects are what allow PNP to fit data under so many conditions from so many channels. Thus ignoring these effects is likely to lead to qualitative errors in understanding (Eisenberg, 1996). It is important to add that the existence of these effects (and their approximate size) does not depend on details or assumptions of the PNP model.

3. Barrier models of channels assume a prefactor that is independent of the type of ion, particularly of its diffusion coefficient. In fact, traditional barrier models of channels use a prefactor that is different from that derived, simulated, or measured experimentally in condensed phases. The traditional prefactor has no dependence on the type of permeating ion, its friction, or on channel length, and it has the wrong dependence on temperature. These dependencies are not just theoretical constructs; they have been measured by chemists in much experimental work on barrier crossing in condensed phases.

Thus it seems unwise to use barrier models (with the traditional prefactor) as they have often been used, namely, to compare the permeation of different ions, unless one has evidence that different ions experience the same friction and have other identical properties, as discussed previously.

4. Traditional barrier models use the wrong numerical value for the prefactor. For CRCs the traditional prefactor is numerically too large by a large factor, 2.2×10^4 for K^+.

5. Barrier models predict much less current than flows in most open channels, if they use the correct prefactor and the barrier is higher than $\sim 3kT/e$. The conductance of the traditional model of CRC, predicted using the correct prefactor, using a parabolic barrier $3kT/e$ high, and using the parameters of the CRC channel reported here, is some 2 psiemens, in 100 mM KCl, much less than the hundreds of picosiemens we find. Evidently barriers are low in most open channels. If this is so, traditional barrier theory makes no sense.

6. Barrier models describe the effects of mutations in channel proteins only vaguely because they do not include Poisson's equation (or, equiva-

lently, Coulomb's law applied to all charges) to show how a mutation in a protein, which often changes the fixed charge lining the wall of the protein's pore, changes the potential profile, barrier height, or rate constant for flux.

Given these difficulties, it is not surprising that barrier models of channels are unable to fit the currents measured in a number of types of channels (if measurements are made over a wide range of potentials and in a wide range of solutions) and that they are of quite limited use in understanding the general phenomena of selectivity or the specific effects of mutations in channel proteins.

CONCLUSION

It seems to us that the time has come to abandon barrier models of the CRC channel and perhaps of other open channels as well. It seems reasonable to us to see how well PNP can serve as a replacement, by checking its predictions over a wide range potentials, in a wide range of ions and mixtures of ions.

On theoretical grounds, it seems unlikely to us that PNP in its present form will be adequate to this task. Nonetheless, an adequate replacement is likely to preserve PNP's main features, namely, the description of the channel as a distribution of permanent charge, and the calculation of the potential and concentration profiles, and flux, as the self-consistent solution of Poisson and transport equations.

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