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Elimination and Restoration of Voltage Dependence in the Mitochondrial Channel, VDAC, by Graded Modification with Succinic Anhydride

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Summary. The major permeability pathways of the outer mitochondrial membrane are the voltage-gated channels called VDAC. It is known that the conductance of these channels decreases as the transmembrane voltage is increased in the positive or negative direction. These channels are known to display a preference for anions over cations of similar size and valence. It was proposed (Doring & Colombini, 1985*b*) that a set of positive charges lining the channel may be responsible for both voltage dependence and selectivity. A prediction of this proposal is that progressive replacement of the positive charges with negative charges should at first diminish, and then restore, voltage dependence. At the same time, the channel's preference for anions over cations should diminish then reverse. Succinic anhydride was used to perform these experiments as it replaces positively charged amino groups with negatively charged carboxyl groups. When channels, which had been inserted into phospholipid membranes, were treated with moderate amounts of the anhydride, they lost their voltage dependence and preference for anions. With further succinylation, voltage dependence was regenerated while the channels became cation selective. The voltage needed to close one-half of the channels increased in those treatments in which voltage dependence was diminished. As voltage dependence was restored, the voltage needed to close half of the channels decreased. The energy difference between the open and closed state in the absence of an applied field changed little with succinylation, indicating that the procedure did not cause large changes in VDAC's structure but specifically altered those charges responsible for voltage gating and selectivity.

Key Words voltage gating · membrane channel · selectivity · gating mechanism · protein modification · VDAC

Introduction

Cell membranes serve as barriers to the movement of ions and nonelectrolytes. Thus, the regulation of cell membrane permeability is of considerable interest. One way that cells facilitate and control transport is through water-filled pores or channels. Most channels can exist in at least two conductive states: an open (maximally conducting) state and a closed (a reduced or nonconducting) state. When the transmembrane potential influences the probability of finding the channel in a particular conducting state, that channel is said to be voltage dependent.

In theory, voltage dependence could arise from the movement of charges (gating charges) along an electric field or the alignment of dipoles with an electric field (Hodgkin & Huxley, 1952). This movement is relative to the field and may result in whole or part from movement of the electric field (Colombini, 1984). The ions or dipoles act as voltage sensors allowing the channel to detect and respond to a change in the transmembrane voltage. The position of the sensors relative to the field act to stabilize or destabilize a particular channel conformation. This results in a change in the probability of finding the channel in a particular conducting state. The molecular events underlying voltage dependence are poorly understood.

It has been known for many years that the outer mitochondrial membrane is a permeability barrier (Werkheiser & Bartley, 1957). The presence of pores was suggested by stain-filled pits in electron micrographs of negatively stained membranes (Parsons, Bonner & Verboon, 1965; Parsons, Williams & Chance, 1966). An outer membrane polypeptide was later identified (Mannella & Bonner, 1975). The presence of outer membrane pores has been demonstrated (Colombini, 1979; Zalman, Nikaido & Kagawa, 1980; Freitag, Neupert & Benz, 1982; Roos, Benz & Brdiczka, 1982). Recent work has identified the stained pits on mitochondrial outer membranes as VDAC, the voltage dependent, anion-selective channel (Mannella & Colombini, 1984). VDAC has been found in all mitochondrial outer membranes examined to date (in protozoans by Schein, Colombini & Finkelstein, 1976; in fungi by Colombini, 1980*a*; Freitag et al., 1982; in higher plants by Zalman et al., 1980; Smack & Colombini, 1985; and in mammals by Colombini, 1979; Linden, Gellerfors & Nelson, 1982; Roos et al., 1982; Linden & Gellerfors, 1983). After VDAC's insertion into planar lipid

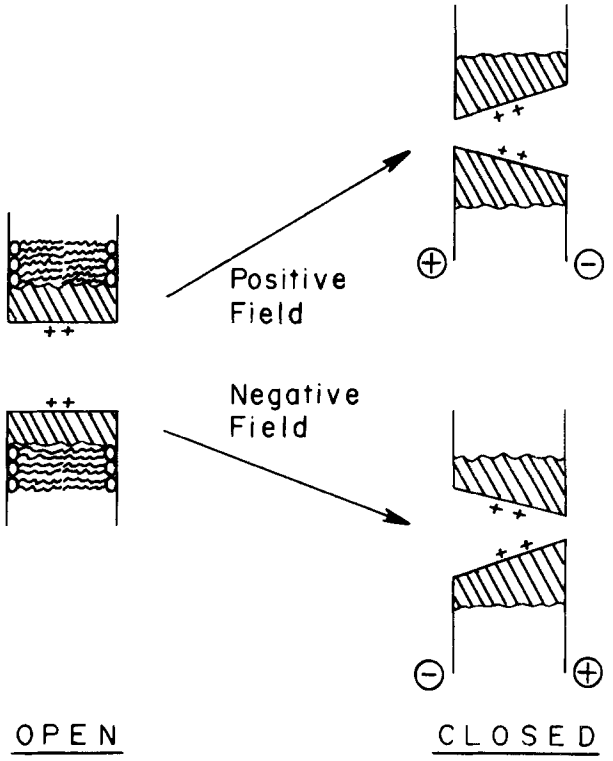


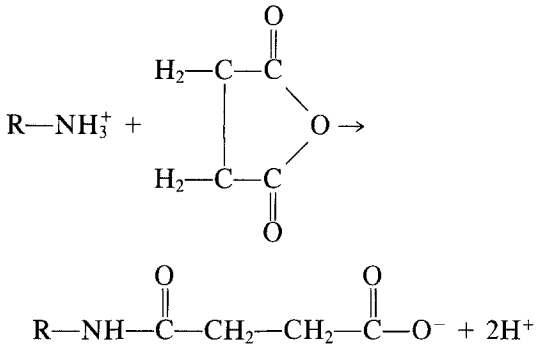
Fig. 1. Proposed mechanism for voltage gating in the mitochondrial channel, VDAC. A set of positive charges line the interior of the pore (the number of charges represented in the Figure has no relation to the actual number of charges in the interior). It is proposed that these charges are responsible for the phenomena of voltage dependence and anion selectivity (reprinted from Doring and Colombini, 1985*b*)

bilayers, its characteristic voltage dependence and anion selectivity may be examined (Schein et al., 1976).

As the transmembrane voltage is increased in the positive or negative direction, VDAC's conductance decreases. This reduction in conductance is the result of channel closure. As the transmembrane voltage is increased the probability of finding VDAC in the maximally conducting state is reduced.

In addition to its voltage dependence, VDAC shows a preference for anions over cations of similar size and valence (Schein et al., 1976). VDAC's pore radius has been estimated at 2.0 nm (Colombini, 1980*a*), although others have reported the smaller estimate of 1.0 to 1.5 nm (Mannella, 1982). In view of this large pore diameter relative to the size of the ions, it seems likely that fixed positive charges lining the pore interior or near the pore mouth are acting as selectivity filters.

Recent evidence suggests that lysine epsilon amino groups are largely responsible for VDAC's voltage dependence (Bowen, Tam & Colombini, 1985). These groups are positively charged at pH 7.5. Titration by increasing pH renders the channel voltage insensitive (Bowen et al., 1985). Succinic anhydride, which converts positive amino groups to carboxyl groups (Habeeb, Cassidy & Singer, 1958), also diminishes VDAC's voltage dependence (Doring & Colombini, 1985*a*). In addition, succinic anhydride reduces, and with larger additions reverses, VDAC's preference for anions over cations (Doring & Colombini, 1985*a*). Thus it appears likely that the same set of charges may be responsible for the dual functions of voltage sensor and selectivity filter (Doring & Colombini, 1985*b*).



The aim of the work reported here was to test a prediction made by the current working hypothesis for VDAC's voltage-gating mechanism. In this mechanism (Doring & Colombini, 1985*b*) the positive charges lining the channel are responsible for VDAC's voltage dependence and anion selectivity (Fig. 1). If this model is correct, substitution of negative charges for the positive charges, should initially diminish VDAC's voltage dependence. As more of the charges are modified, voltage dependence should be restored. In addition, modification should alter VDAC's selectivity from a preference for anions to a preference for cations. For this study various amounts of succinic anhydride were added to a salt solution bathing a phospholipid bilayer containing VDAC. The voltage dependence and open-channel selectivity were determined before and after reaction with succinic anhydride. The selectivity (estimated by measuring the reversal potential in the presence of a transmembrane salt gradient) was used as a measure of the degree of anhydride modification. By following how the steepness of the voltage dependence [as measured by the parameter n (*see below*)] varied with the degree of channel modification, the voltage-gating mechanism could be tested.

Materials and Methods

VDAC Isolation

Outer mitochondrial membranes of a wall-less mutant of *Neurospora crassa* (FGSC 326) were isolated as described by Mannella (1982). The outer mitochondrial membranes were suspended in a solution of 1 mM KCl, 1 mM Tris-HCl, at pH 7.5 to an approxi-

mate protein concentration of 2.5 mg/ml. Dimethylsulfoxide (DMSO) was added to a final concentration of 15% (vol/vol) and the solution was stored at −20 or −70°C.

The outer mitochondrial membranes were combined with asolectin to make a lipid-membrane suspension for use in bilayer formation (as per the method of Schein et al., 1976). Briefly, DMSO was removed from the outer mitochondrial membrane suspension by diluting with a 15-fold excess of 1 mM Tris-HCl, 1 mM KCl, at pH 7.5 and centrifuging at 15,000 × *g* for 20 min at 4°C. The outer membranes were combined with an excess of asolectin (1 mg protein to 20 mg asolectin) and suspended in 1 mM Tris-HCl, 1 mM KCl, at pH 7.5 to a final lipid concentration of 1% (wt/vol). The solution was then sonicated under N_2 in an ice bath for 2 to 3 min until somewhat clarified. The lipid-membrane mixture was then divided into 0.5- to 1-ml aliquots, lyophilized, and stored in a desiccator at −20°C.

To prepare for use, the lipid-membrane powder was brought up to a 1% suspension (wt/vol) in hexane. At this point extra lipid was often added to the lipid-membrane suspension in an attempt to limit channel numbers. Before use, the soybean phospholipids were purified as described by Kagawa and Racker (1971).

Experimental

Planar phospholipid membranes were made according to the method of Montal and Mueller (1972) as modified by Schein et al. (1976). A phospholipid bilayer was constructed across a hole (0.1 to 0.15 mm in diameter) in a Saran partition separating two aqueous compartments labeled *cis* and *trans*. The following constraints resulted in the particular experimental design chosen: 1) In order to achieve high degrees of modification with succinic anhydride, buffer must be added and the net result is an increase in the ion concentration. In order to minimize the impact of these ions on the reversal potential, succinic anhydride was only added to the *cis* side (the high-salt side). It had been shown (Doring & Colombini 1985*a*,*b*) that succinic anhydride added asymmetrically to the VDAC channels, in a planar membrane, resulted in the loss of voltage-gating at both positive and negative potentials. Therefore it was not necessary to add anhydride to both sides of the membrane. 2) Since anhydride was only added to the *cis* side and from time to time channels insert into the membrane during an experiment, VDAC-containing phospholipid solution was layered only on the *cis* side (*see below*) while protein-free phospholipid solution was layered on the *trans* side. 3) In the presence of a salt gradient, voltage-dependent closure of VDAC is easily observed when the high-salt side is made negative but much harder to detect when the high-salt side is made positive. The reasons for this are the reversal potential of the channels and the selectivity change when VDAC closes. The selectivity of the open state is weakly anion selective while that of the closed state is essentially nonselective. Therefore, when the high-salt side is made positive there is only a small change in current resulting from channel closure. It was mainly for this reason that voltage-dependence was monitored as described below. 4) Additions of the large amounts of anhydride needed to restore the voltage-dependence, tended to destabilize the membrane. Therefore a stimulating protocol was chosen which reduced the time needed to collect the data. Asymmetrical triangular voltage waves were used in which *dV/dt* was low in the direction of decreasing electric field and high in the direction of increasing field (the shape approximated a sawtooth). In addition, the voltage range examined was limited to the minimum region essential to analyze the channel's voltage dependence and selectivity.

The *cis* compartment contained 1 M KCl, 5 mM $CaCl_2$ and 5 mM of buffer (Tris-HCl or K-HEPES) pH 7.5. The 1% lipid-membrane suspension (10 to 20 μl) was layered on the *cis* solution. The *trans* compartment contained 100 mM KCl, 5 mM $CaCl_2$ and 5 mM buffer, pH 7.5. A lipid solution (1% asolectin in hexane) was layered on the *trans* solution. After hexane evaporation, the planar bilayer was made from the monolayers by injecting solution into the sub-phase. Once formed, the membranes usually contained VDAC (as described by Schein et al., 1976). Each compartment contained approximately 4 ml of aqueous solution.

An operational amplifier was used to clamp the voltage across the membrane and monitor current flow (Schein et al., 1976). Calomel electrodes were used to interface with the salt solutions. The *trans* side of the chamber was maintained at virtual ground. Current flow through the membrane was recorded with a Kipp and Zonen BD41 dual-pen chart recorder. Asymmetric triangular waves were generated by a Wavetek (model 184) function generator and applied to the *cis* side of the membrane.

VDAC's voltage-dependent properties were characterized in the presence of the 10-fold gradient of KCl by means of asymmetric triangular voltage waves [one ramp was steep (high *dV/dt*) while the other was shallow (low *dV/dt*)]. In the control experiments the voltage changed linearly from −85 mV to approximately 25 mV. From experiment to experiment the frequency was varied over the range 2 to 4 mHz. The rate of voltage change was such as to ensure the measurement of steady-state conditions for channel opening. The voltage-dependent parameters of the channels were determined as described below. The reversal potential of the open channel was the voltage necessary to bring the current flowing through the channel to 0.

Various amounts of succinic anhydride dissolved in DMSO were added to the *cis* side of the membrane. (DMSO alone had no effect on VDAC's voltage dependence or ion selectivity. In addition, DMSO did not affect the conductance of the lipid bilayer.) During the time of succinic anhydride addition the transmembrane potential was maintained at 0 mV. The pH dependence of the anhydride reaction with amino groups (it reacts with the uncharged form) requires the medium pH to be maintained above 7. Therefore, if the amount of succinic anhydride to be added to the aqueous phase was such as to decrease the pH of the compartment below 7.0, additional buffer in the form of $KHCO_3$ was added to the *cis* compartment before the addition of the succinic anhydride.

In a typical experiment, VDAC was inserted into a planar membrane and its voltage dependence and reversal potential were measured. After the addition of succinic anhydride, these measurements were repeated. If the addition of succinic anhydride resulted in a change of the sign of the open-channel reversal potential, the polarity of the applied asymmetric voltage wave was reversed. The polarity of the applied wave was chosen so as to maximize the driving force on the salt flowing through the channel. In this way, the decrease in the conductance with channel closure was evident. At times, successive additions of anhydride could be made on the same membrane.

Data Analysis

The measurements of current as a function of voltage were digitized using a Hewlett-Packard 85 computer. A modification of the method described for Excitability Inducing Material channels (EIM) by Ehrenstein, Lecar and Nossal (1970) and for VDAC by Schein et al. (1976) was used for analysis of the channel's voltage dependence. It was assumed that the channels could exist in 1 of only 2 conducting states, open and closed. Although it is known that VDAC channels exist in more than two conducting states (Colombini, 1980*b*), the two-state approximation was used for the following reasons: (1) The results of the analysis applied to

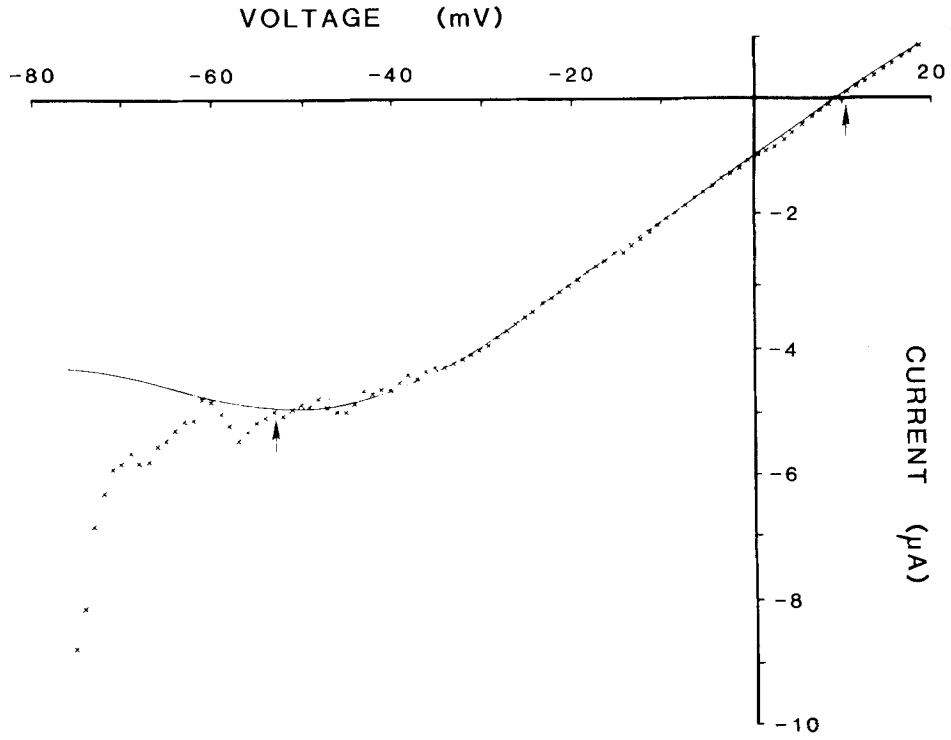


Fig. 2. Current-voltage relationship obtained experimentally and determined theoretically. The experimental values (indicated by ×) were obtained from a membrane with many channels to which no succinic anhydride had been added. The theoretical *I-V* curve is represented by the unbroken line. The theoretical curve was derived from the equation: $I = N_oG_o(V\text{-}E_o) + N_cG_c(V\text{-}E_c)$, with an n of 2.5, a nFV_o of 14,000 joules and an E_c of 0 mV (*see* Data Analysis). The experimental *I-V* values between the arrows were used for comparison with the theoretically derived values

VDAC are consistent with results obtained from the two-state system. (2) Because the channels exist in many closed states, a mathematical analysis of such a system would be extremely difficult. (3) While the exact meaning of the voltage-dependent parameter values may be questioned, these values are a valid measure of the steepness of the voltage dependence regardless of the number of closed states.

In order to characterize the voltage dependence of the modified channels, theoretical current-voltage curves were generated by varying the parameters n, nFV_o, and E_c (E_o was directly measured) and these were fitted to the curves obtained experimentally. n is a measure of the steepness of the voltage dependence and is equal to the number of charges which would have to move through the electric field, as the channel changes conductance states, to account for the voltage dependence. nFV_o is the energy difference between the open and closed states in the absence of an applied field. When the applied voltage equals V_o the energy levels of the open and closed states are equal. E_c is the reversal potential of the "closed" channel (this state still conducts ions but at a much lower level than the open state). The sum of the squares of the difference between the experimental current values and theoretical values was minimized. The theoretical current values were derived from the equation:

$$I = G_oN_o(V - E_o) + G_cN_c(V - E_c) \quad (1)$$

where E_o and E_c are the reversal potential of the open and closed channel, respectively. G_o is the open-channel conductance and G_c is the closed-channel conductance. N_o is the number of open channels, N_c is the number of closed channels, and Nt is the total. The maximum and minimum conductances were related to Nt by:

$$G_oNt = G_{max} \quad (2)$$

$$G_cNt = G_{min} \quad (3)$$

The equation relating the number of open and closed channels at any voltage V is based on the Boltzmann distribution.

$$N_o = Nt/(\exp(nF(V\text{-}V_o)) + 1). \quad (4)$$

where F, R and T are Faraday's constant, the gas constant, and the temperature (in degrees Kelvin), respectively.

Figure 2 shows a set of typical experimental values superimposed on a theoretical *I-V* curve generated with n = 2.5, nFV_o = 14 kJ and E_c = 0 mV. Indicated on the Figure is the region over which the two curves were compared. Regions of channel closure were excluded from analysis because they represent a nonsteady state situation[1]. The value of E_o was directly measured for each experiment. The G_{max} was measured and then

[1] The current values recorded at high potentials were higher than the steady-state current because of the following: a) the rising phase of the voltage stimulus was operated at high dV/dt; b) the slow closing kinetics of VDAC closure. Thus channel closure was still occurring at this time and therefore these points were excluded from the calculations.

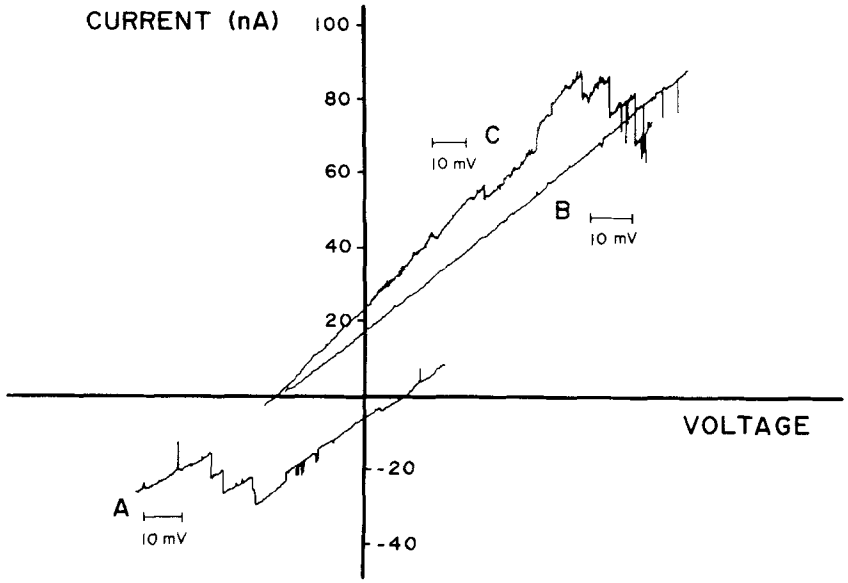


Fig. 3. Current-voltage relations of control and modified channels. The channels in Trace *A* were unmodified. In Trace *A* the voltage increased from −60.5 to 21.0 mV at a rate of 0.46 mV/sec. Trace *B* is the current-voltage relation after the addition of 1 mg of succinic anhydride in DMSO to the solution bathing the *cis* side of the membrane. In Trace *B* the voltage decreased from 76.6 to −18.7 mV at a rate of 0.44 mV/sec. Trace *C* was obtained after an additional 7 mg of succinic anhydride had been added to the *cis* compartment. In Trace *C* the voltage decreased from 87.5 to −29.1 mV at a rate of 0.53 mV/sec

normalized to 1. The G_{min} could not usually be measured from the current-voltage plot because typically not all the channels closed. In addition, the presence of the salt gradient introduced a new variable E_c, and shifted the V_o to higher voltages. Thus, the G_{min} was estimated from separate experiments in which a voltage pulse (of sufficient magnitude to close virtually all the channels), was by applied and the resulting conductance expressed as a percent of the G_{max} (45% of G_{max} or 0.45 for the normalized G_{max}).

Results

VDAC-containing planar membranes were made and the current through these channels monitored as a function of voltage. In this way a control *I-V* relation was obtained. From this relation, the steepness of the voltage dependence as well as the reversal potential of the open, unmodified, channel were determined. Succinic anhydride was added to the solution bathing the *cis* side of the membrane. Succinic anhydride reacts quickly with water (with an estimated half-life of 2 min) to form succinate. This results in variability in the amount of modification with a given addition. To compensate for this variability, the change in the open-channel reversal potential was used as a measure of the extent of the reaction. After modification, *I-V* relations were again obtained and used to determine the open-channel reversal potential and degree of voltage dependence.

An example of the type of data collected is shown in Fig. 3. Trace *A* shows a current-voltage relationship derived from a membrane containing four unmodified VDAC channels. The number of channels was determined from the maximum conductance of the wave divided by the single-channel conductance. The opening of the channels is reflected in the abrupt changes in current. The reversal potential of the open channels was 10.5 mV. Trace *B* shows a current-voltage relationship obtained from the same membrane after the addition of 1 mg of succinic anhydride to the aqueous phase bathing the *cis* side of the membrane. The reversal potential changed to −19.2 mV, indicating a change in the selectivity of the channel. Because the sign of the reversal potential has changed, the polarity of the applied wave was reversed. The current-voltage trace at this stage of modification was essentially ohmic, although the voltage applied reached 76.6 mV. (Succinate had no effect on VDAC's selectivity or ability to close.) Thus, as reported previously, the voltage dependence of the channels was eliminated (Doring & Colombini, 1985*a*). Trace *C* is the current-voltage relation after the addition of an additional 7 mg of succinic anhydride to the *cis* aqueous phase. The reversal potential has reached −25.2 mV. Channel closure was again evident, indicating that voltage dependence had been restored.

In several experiments, multiple additions of succinic anhydride were made. Figure 4 shows the voltage dependence, as measured by the parameter *n* (*see* Data Analysis) as a function of open-channel reversal potential. This graph was generated from *I-V* relations obtained from five additions of succinic anhydride to the same membrane. The membrane contained four channels. With increasing modification the reversal potential was changed from the positive control value to negative values. The voltage dependence *n*, decreased from the control value of 2.8 to a low of 0.4. With increasing modification, *n* increased to a value of 1.8.

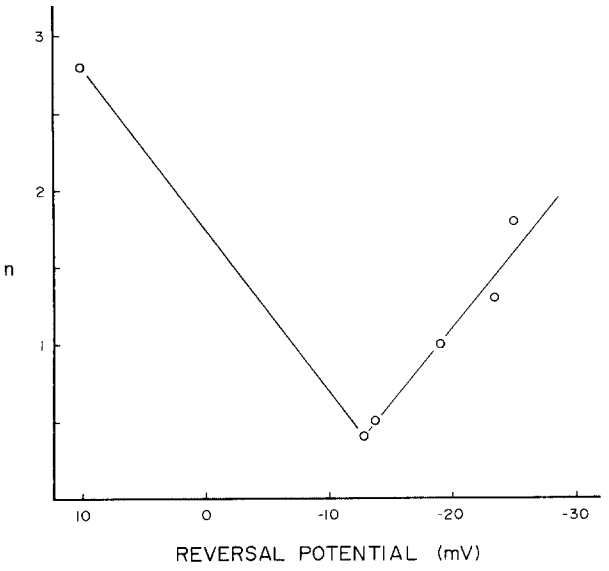


Fig. 4. Steepness of the voltage dependence, n, as a function of open-channel reversal potential. The data were generated from the analysis of the results of five successive additions of succinic anhydride to the *cis* compartment bathing the same membrane. The membrane initially contained four channels although there was evidence of the insertion of an additional channel after modification

Voltage dependence versus reversal potential obtained from a series of experiments is represented in Fig. 5. The membranes contained between 2 and 50 channels. In order to eliminate the variability contributed by differing degrees of unmodified channel closure, voltage dependence was normalized. For each experiment a measure of the control channel voltage dependence n was obtained. The experimentally determined values of n were normalized by dividing by the corresponding control value of n. Voltage dependence decreased as the reversal potential became negative. In the group represented by the average reversal potential of -17.7 mV (-15 to -20 mV), the normalized n reached a minimum. At the average reversal potential of -17.7 mV, the mean voltage dependence was 14% of the control. With further modification of the channel, as indicated by the continued decrease in the reversal potential, voltage dependence was regenerated. With the greatest degree of modification, indicated in the group with the average reversal potential of -34.3 mV, voltage dependence was restored to a value of 70% of the control.

Figure 6 shows V_o, the voltage at which half the channels are open, as a function of open-channel

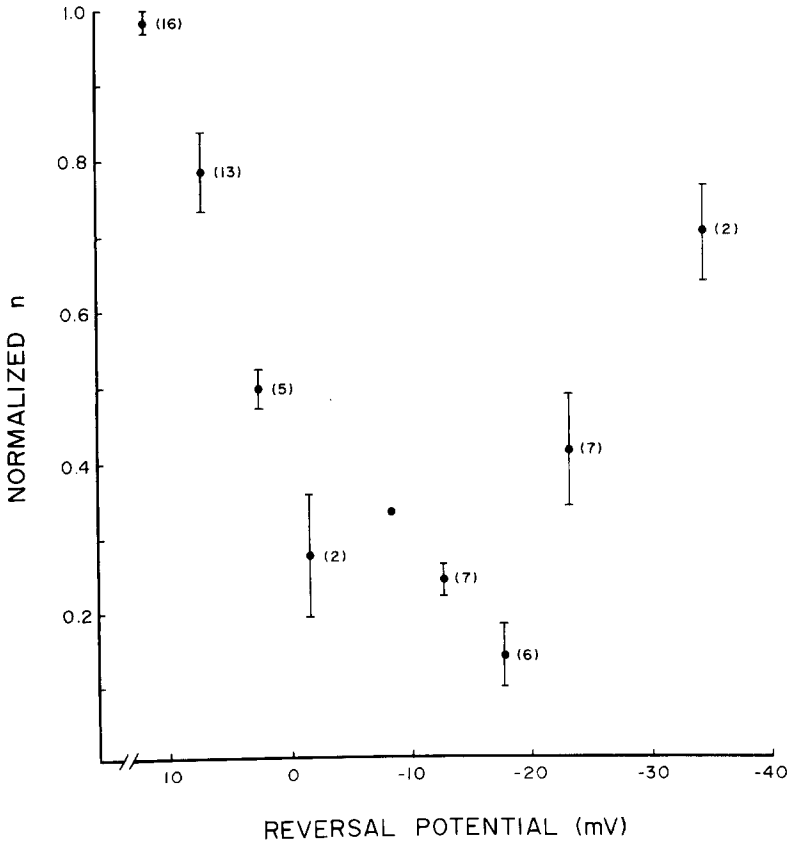


Fig. 5. Normalized n as a function of open-channel reversal potential. The n's were normalized by division by the corresponding control value of n. The data were combined with respect to open-channel reversal potential in 5-mV increments. The normalized n of the group, with standard error of the mean, is plotted versus the average open-channel reversal potential of the group. The number of observations in each group are in parentheses. The data were obtained from 21 membranes, with multiple additions of succinic anhydride to many membranes. (A single observation is represented by the lone point.)

reversal potential. In those groups in which the normalized n was decreasing (as indicated in Fig. 4), V_o was increasing. When normalized n was at a minimum (as found in the group with the reversal potential of -17.7 mV), V_o reached its maximum mean value of 860 mV. As voltage dependence was regenerated, V_o decreased. When voltage dependence had regenerated to 70% of the control value, V_o had decreased to a value of 63.9 mV (122% of the V_o of the unmodified channels). The increase in V_o with decreasing normalized n, is consistent with the modification of voltage sensors. As the positive sensors are replaced with negative charges, the channel is less able to sense and respond to an applied voltage. Concurrent with the loss of the ability to respond to a voltage, is the necessity of applying an increased voltage in order to close one-half of the channels. As net charge in the channel was restored and voltage dependence was regenerated, the voltage necessary to close one-half of the channels decreased.

Statistical tests were used to assess the significance of the changes in voltage dependence with increasing modification. For these tests, the open-channel reversal potential was normalized in order to further reduce the variability between experiments. In Fig. 7, normalized n as a function of normalized open-channel reversal potential is represented. The lower panel shows the individual normalized values of n plotted versus normalized reversal potentials. In the upper panel the data were grouped per every 0.4 normalized reversal potential units. The control group mean was significantly different from the means of the other groups. In short, the means of all the groups in which the normalized n's are decreasing are significantly different from each other. The only exception is group E versus group F. The mean of the group with the minimum normalized n is significantly different from the means of all those groups in which voltage dependence is regenerated (as indicated by an increase in normalized n). The means of groups H and I (where voltage dependence is increasing) are significantly different from the mean of group J (in which restoration of voltage dependence is maximized).

Experiments using membranes with only a few channels indicated what may be happening on the molecular level. Figure 3 shows that with intermedi-

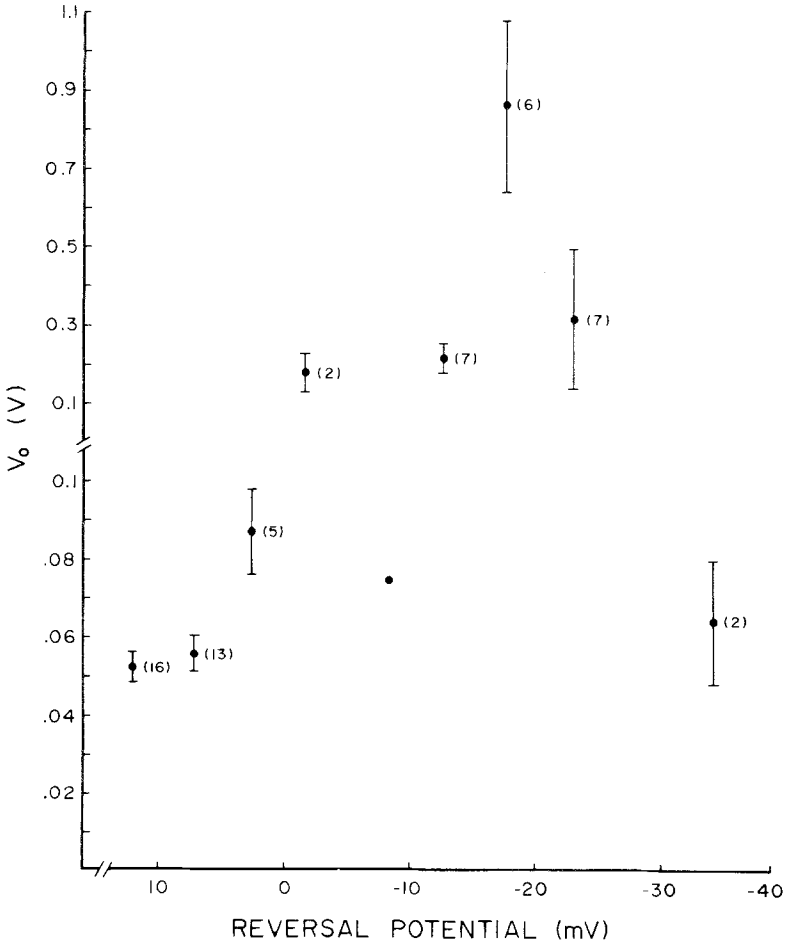


Fig. 6. The voltage at which half the channels were closed V_o as a function of open-channel reversal potential. These values were determined from the fitted values of n and nFV_o. In records showing low-voltage dependence, the fitting process was poorly sensitive to changes in nFV_o. This resulted in increased variability in the corresponding V_o values. The data were combined with respect to reversal potential in 5-mV groupings. The mean V_o, with standard error of the mean, is plotted as a function of the average open-channel reversal potential of the group. The data were collected from experiments obtained from 21 different membranes (some membranes yielded multiple observations). The number of observations in each group is shown in parentheses. (A single observation is indicated by the lone point.)

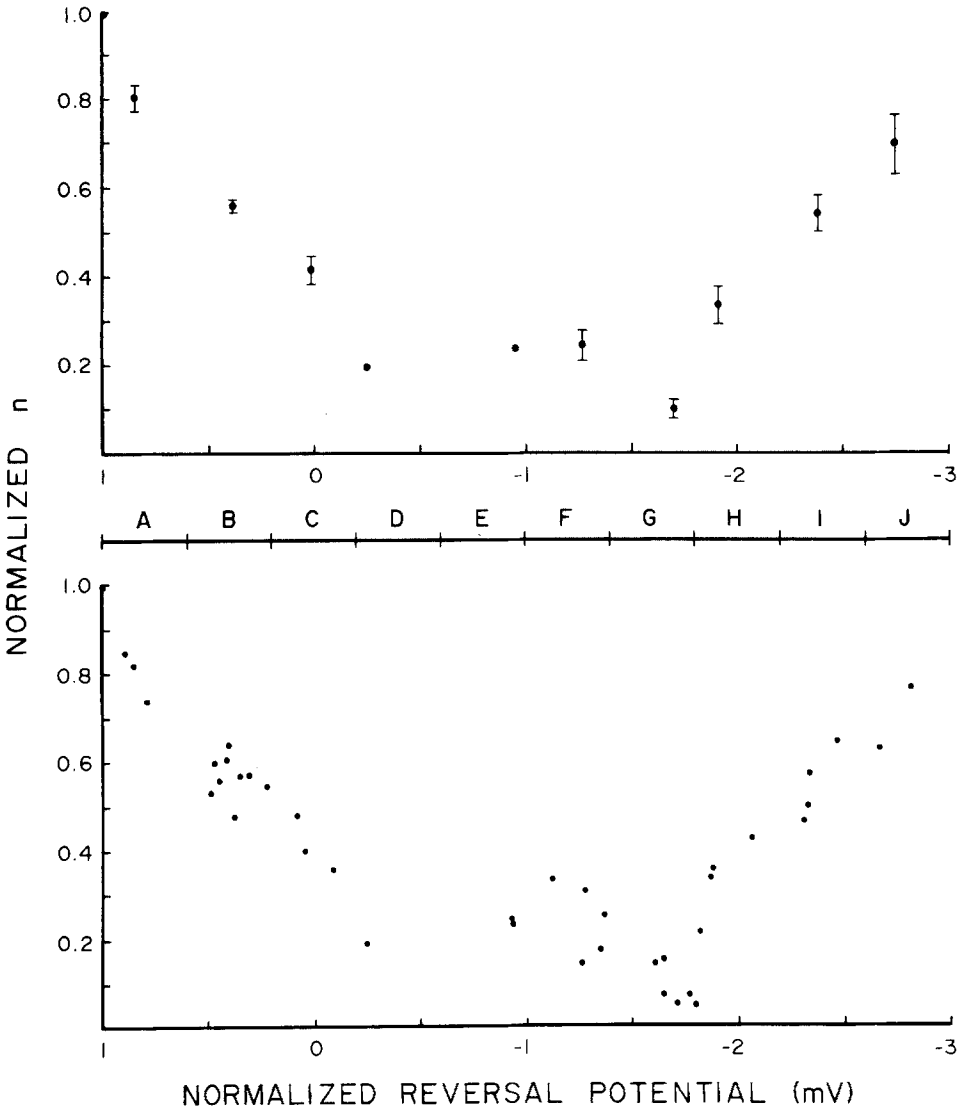


Fig. 7. Normalized n as a function of normalized reversal potential of the open channel. In the lower panel normalized n is plotted as a function of normalized open-channel reversal potential. The experimental points were obtained from 21 membranes. In the upper panel the data were combined with respect to normalized reversal potential in increments of 0.4 normalized reversal potential units. The normalized n, with standard error of the mean, is plotted as a function of the average normalized open-channel reversal potential. The standard error of E was within the dot. The difference in group means was tested for level of confidence. Relevant comparisons are listed below.

Group	*vs.*	Group(s)	Confidence Level
Control		A,B,C,E,F	99.5%
A		B,C,E,F,G	99.5%
B		C,E,F,G	99.5%
C		E,F	97.5%
C		G	99.5%
E		G,I	97.5%
E		J	99.5%
F		G,I,J	99.5%
G		H,I,J	99.5%
H		J	99.0%
I		J	95.0%

ate modification none of the channels closed[2]. With increasing succinylation of the channel, voltage dependence returned. The change in voltage dependence with modification is a reflection of the probability of channel closure with a given voltage. In those channels with intermediate levels of modification (with reversal potential between −10 and −20 mV) there is a very low probability of channel closure, even with applications of voltages as large as 80 mV (which closes virtually all unmodified channels). With increasing modification (reversal potentials more negative than −22 mV) the probability of channel closure increased.

The ion selectivity of the VDAC channels was estimated from the reversal potential measurements using both the electroneutrality and constant field assumptions since it is not clear which assumption best approximates the conditions found within large channels. In Table 1 the calculated permeability ratios of anions to cations and the corresponding representative reversal potentials are reported. These reversal potentials correspond to the mean reversal potentials obtained in the groupings represented in Figs. 5 and 6. The channels' preference of Cl^- over K^+ ions decreased with succinylation until the channel strongly preferred cations.

Table 1. Succinic anhydride modifies VDAC's ion selectivity

Reversal potential (mV)	Permeability ratio	
	Cl^-/K^{+a}	Cl^-/K^{+b}
12.0	1.6	1.9
7.2	1.4	1.5
2.6	1.2	1.2
−1.6	.92	.90
−8.4	.72	.65
−12.7	.60	.52
−17.7	.49	.39
−23.2	.38	.29
−34.3	.20	.13

[a] Mobility ratio according to electroneutrality (Nernst-Planck) approximation.
[b] Permeability ratio as determined by constant field (Goldman-Hodgkin-Katz) approximation.

Discussion

The ability of certain membrane proteins to respond to changes in membrane potential by changing their conformation and therefore their activity, is a very important biological phenomenon that is essential for the function of many systems. However, the molecular basis for this phenomenon is poorly understood especially for endocytic channels (i.e. channels that are produced by cells for insertion into their own membranes). The mechanisms used by ectocytic channels (those that enter a cell's membrane from the environment) may not function in the endocytic channels because the voltage probably does not drive these channels into the membrane.

For the mitochondrial channel, VDAC, a molecular mechanism has been proposed (Doring & Colombini, 1985*b*) which accounts for a great many observations made on this channel (Bowen et al., 1985; Colombini, 1986). This mechanism (Fig. 1) proposes that a set of positive charges on the protein are located on the walls of the protein's aqueous pore. This group of charges serves both as a voltage sensor and as a selectivity filter. Since no receptor or binding site is proposed, the mechanism is simple. The electric field exerts a force on these charges which is transmitted to a critical portion of the protein resulting in a conformational change which partly occludes the channel. The presence of a net charge is all that should be needed in order for the electric field to be able to exert its force. If this is true then one should be able to change the charge on this sensor and change its voltage dependence. Indeed, if the sign of the charge were reversed, the voltage gating should still function. This work has confirmed this prediction. Low levels of anhydride modification should have produced an essentially neutral sensor by converting some of the positive charges into negative charges. High levels of modification should have resulted in a sensor with a net negative charge thus restoring the voltage dependence.

As the chemical modification caused the channel to lose its ability to sense the transmembrane potential, the voltage of transition between the open and closed state increased. V_o, the voltage at which one-half of the channels are closed, increased with a decrease in n. This observation is cardinal to the thesis that VDAC's voltage sensors are modifiable charges. As voltage dependence was regenerated, with an increase in n, V_o again decreased. As the voltage-sensing ability of the channel was lost, a higher applied voltage was necessary to close half of the channels. As voltage dependence was restored, the voltage necessary to close half of the channels decreased. If succinylation was not directly modifying the charges on the sensor, but rather affecting the field felt by the voltage sensor or

[2] Although the polarity of the applied voltage has been changed, VDAC's voltage-dependent properties are quite symmetrical around 0 (Schein et al., 1976; Colombini, 1986).

stabilizing one particular conformation, V_o would shift without a change in the voltage dependence of the channels. Such shifts in V_o have been reported in other systems (Huang, Moran & Ehrenstein, 1982; Hahin & Campbell, 1983; Hanke & Miller, 1983).

The energy difference between the open and closed state in the absence of a field is equal to nFV_o. If succinylation affects only VDAC's gating charges, no change in nFV_o with modification is expected. The correlation coefficient of nFV_o with open-channel reversal potential is -0.25. This is significant at the 95% confidence level. However, the mean nFV_o of the unmodified channels was not significantly different from the mean nFV_o in those groups in which channels had been highly modified. The small decrease in nFV_o with increasing modification (as indicated by the correlation coefficient) may indicate some minor structural modification of the channel. However, this effect is small in comparison to the effect of succinic anhydride on voltage dependence and selectivity.

An alternate interpretation of the change in VDAC's conductance-voltage relation with modification is a change in the closed conductance of the modified channel. If the minimum conductance of the channels was increased with modification, voltage dependence would appear to decrease. However, this interpretation is inconsistent with the increase in voltage dependence in those channels which have been highly modified. Additional evidence refuting this interpretation is the essentially unchanged single-channel closed conductance between the native and highly modified channel (46 ± 2% and 49 ± 3% of the maximum conductance, respectively). Therefore succinylation of the channels did not significantly affect the closed channel's conductance as a percent of the open-channel conductance. Likewise, the conductance of the open channels was essentially unchanged at 1.8 nS. Those channels with decreased n's spend more time in the open state at any given voltage.

The proposed mechanism attributes a dual function to the charged sensor. The mechanism predicts that altering the charge on the sensor should alter ion selectivity and voltage dependence in a parallel manner. This was indeed the case as demonstrated by the good correlation between voltage dependence (as measured by the parameter n) and ion selectivity (as measured by the reversal potential). This correlation could be coincidental resulting from the simultaneous modification of two sets of charges, one being involved in responding to the electric field and the other in ion selectivity. Indeed, the minimum degree of voltage dependence was not observed at 0 reversal potential. In addition, high degrees of modification result in only partial recovery of the voltage dependence (Figs. 5 and 7) while the newly formed cation selectivity is much greater than the original anion selectivity (Table 1). However, it is not uncommon to find uncharged channels which are ion selective so that other factors, in addition to net charge, do influence ion selectivity. It is also possible that the walls lining the aqueous pore have both positive and negative charges but that an excess of positive charges exists resulting in the weak anion selectivity of the unmodified channel. Modification would result in a much stronger final negative charge and thus a higher selectivity. Succinylation adds a 0.6-nm arm so that the new charges probably penetrate more toward the center of the pore (important in the high ionic strength environment) resulting in a greater influence on ion selectivity. Finally, if only some of the charges in the pore are involved in the gating process, the observations could easily be accounted for.

In a simple model, VDAC's selectivity is a result of positively charged groups lining the pore (*see* details in Appendix). Assuming electroneutrality (reasonable for a large channel in a high ionic strength medium), these groups will result in a steady-state excess level of free anions in the channel. This excess of free anions over free cations could account for the observed selectivity. Assuming a 5-nm-long cylindrical pore, uniform distribution of fixed charge within the pore, equilibrium at each end with the bulk phase, equal mobility of K^+ and Cl^- within the pore, and electroneutrality, the Nernst-Planck flux equations were solved for the zero current steady state. The reversal potential in the presence of a gradient of KCl (1 M *vs.* 0.1 M) was used to calculate the number of fixed charges in the channel. This was done as a function of anhydride modification. Table 2 shows the results of these calculations for two estimates of channel radius (Colombini, 1980*a*; Mannella, 1982). The amount of calculated charge change from unmodified channel to minimum voltage dependence is the same as the change calculated between minimum voltage dependence and maximal restoration of voltage gating. This is exactly what would be expected if anhydride at first neutralized the charge and then restored it. Since the evidence (Bowen et al., 1985; Doring & Colombini, 1985*b*) indicates that lysine residues are responsible for voltage gating and selectivity, these numbers of positive charges (3 to 6) are consistent with VDAC's lysine content (26 per polypeptide chain, Freitag et al., 1982).

The average control value of n obtained in this work was 2.5 ± 0.6. Thus three lysine groups would be sufficient to account for the voltage dependence. However, n represents the number of charges

Table 2. Calculated number of charges within the pore

Channel status	Reversal potential (mV)	Number of fixed charges channel radius		Change from unmodified channel radius	
		1.5 nm	2.0 nm	1.5 nm	2.0 nm
Unmodified	12	1.4	2.5	—	—
Minimum voltage gating	−15	−1.8	−3.2	3.2	5.7
Voltage gating restored	−35	−4.9	−8.7	6.3	11.2

which move through the entire potential difference in order to account for the voltage dependence and is a minimum estimate of the number of charges on the voltage sensor. A greater number of charges, each moving through part of the applied field with channel closure, would result in the same voltage dependence. The above estimates for the number of charges involved in selectivity (i.e. 3 to 6) are in good agreement with the value of n (3). Thus it is possible that the selectivity filter and voltage sensor are one and the same.

In the current model for VDAC, a set of positive charges line the pore interior. It is these charges which are believed responsible for the dual roles of selectivity filter and voltage sensors. If the current model is correct, increasing reaction of the charges with succinic anhydride should at first diminish and then regenerate the steepness of the voltage dependence. Concurrent with these changes in voltage dependence should be a steady increase in the channels' preference for cations. These predictions have been confirmed in the present investigation.

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Appendix

Calculation of the Number of Fixed Charges Within the Channel

Assumptions:

1) The channel contains fixed positive charges that are uniformly dispersed within the aqueous pore.
2) Electroneutrality is obeyed. Therefore the Cl^- concentration within the channel is equal to the concentration of the fixed positive charge plus the K^+ concentration. Hence: $[K^+]$ in the channel = C, $[Cl^-]$ in the channel = $C + N$, where N is the concentration of fixed charges in the channel.
3) The mobilities of K^+ and Cl^- are equal within the channel (as they essentially are in free solution).
4) Equilibrium is achieved at each mouth of the pore. Therefore:

$$\frac{RT}{F}\ln\left(\frac{Cb1}{C1}\right) = -\frac{RT}{F}\ln\left(\frac{Cb1}{C1+N}\right) = V1 \tag{A1}$$

$$\frac{RT}{F}\ln\left(\frac{Cb2}{C2}\right) = -\frac{RT}{F}\ln\left(\frac{Cb2}{C2+N}\right) = V3\text{-}V2 \tag{A2}$$

where $Cb1$ and $Cb2$ are the bulk concentrations on sides 1 and 2 of the membrane, respectively; $C1$ and $C2$ are the concentrations of K^+ at the mouth of the channel on sides 1 and 2, respectively; $V1$ and $V2$ are the electrical potentials at the mouth of the channel on sides 1 and 2, respectively; zero and $V3$ are the potentials in the bulk phases on sides 1 and 2, respectively; R, T, and F have their usual meanings.

Solution:

Solving the Nernst-Planck flux equations within the channel for an ion gradient and zero current yields:

$$V2\text{-}V1 = \frac{RT}{F}\frac{(-u_- + u_-)}{(u_+ + u_-)}\ln\left(\frac{u_-N + C1(u_+ + u_-)}{u_-N + C2(u_+ + u_-)}\right). \tag{A3}$$

Therefore $V2 = V1$ for equal K^+ and Cl^- mobilities (i.e. $u_+ = u_-$). Substituting into Eqs. (A1) and (A2) and rearranging:

$$\frac{Cb1}{C1}\frac{Cb2}{C2} = \exp\left(\frac{F}{RT}\frac{V3}{}\right) \tag{A4}$$

$$c1 = (-N/2) + (N^2 + 4Cb1^2)^{0.5} \tag{A5}$$

$$c2 = (-N/2) + (N^2 + 4Cb2^2)^{0.5}. \tag{A6}$$

Equations (A4), (A5) and (A6) were solved for the experimental conditions (i.e. KCl bulk activities and measured reversal potentials). N was converted from a concentration to a number of charges assuming a 5-nm-long channel with radii indicated in Table 2.