Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4896–4900, July 1987
Biophysics

Ultrasteep voltage dependence in a membrane channel

(VDAC/channel/excitability/mitochondrion/outer membrane)

PATRICK S. MANGAN AND MARCO COLOMBINI

Laboratories of Cell Biology, Department of Zoology, University of Maryland, College Park, MD 20742

Communicated by William A. Hagins, March 25, 1987

ABSTRACT **A mechanism for regulating voltage-gated channels is presented. The treatment amplifies the effect of the applied membrane potential resulting in a dramatic increase in the channel's voltage dependence. Addition of a large polyvalent anion to the medium bathing a phospholipid bilayer containing the voltage-dependent channel from the mitochondrial outer membrane, VDAC, induced up to a 12-fold increase in the channel's voltage sensitivity. The highest polyvalent anion concentration tested resulted in an *e*-fold conductance change for a 0.36-mV change in membrane potential. On the low end, a concentration of 2 μM resulted in a 50% increase in VDAC voltage dependence. A mechanism based on polyvalent anion accumulation in the access resistance region at the mouth of the pore is consistent with all findings. Perhaps the voltage dependence of voltage-gated channels is amplified *in vivo* by polyvalent ions. If so, the control of excitable phenomena may be under much finer regulation than that provided by membrane potential alone.**

Excitability is a phenomenon important for many physiological processes, including nerve conduction, muscle excitation, hormone release, and egg fertilization. In their investigations into the basis for excitability in the squid giant axon, Hodgkin and Huxley (1) demonstrated the existence of separate voltage-dependent conductances for sodium and potassium ions that were responsible for the generation of action potentials. Subsequently, the planar bilayer and patch-clamp techniques have revealed that voltage-dependent conductances are the result of voltage-gated channels. In these channels, the membrane potential determines the probability of the channel existing in a particular conducting state. The steeper the voltage dependence, the more responsive the cell is to small changes in the membrane potential. The probability of finding the sodium and potassium channels of the squid giant axon in a high conducting state increases *e*-fold for 4 and 5–6 mV, respectively (1). Concerning this voltage dependence, Hodgkin and Huxley (1) noted: "One of the most striking properties of the membrane is the extreme steepness of the relation between ionic conductance and membrane potential." We now report a mechanism that steepens the voltage dependence of the voltage-gated channel of the outer mitochondrial membrane, VDAC. The channel's voltage dependence is increased to values up to an order of magnitude greater than those observed in squid sodium and potassium channels.

VDAC is the major permeability pathway through the outer mitochondrial membrane (2–9). Each channel is in a highly conductive (open) state at zero and low membrane potentials but is converted to a low conducting (closed) state at higher (>20 mV) positive or negative potentials (10). The voltage dependence of VDAC is normally an *e*-fold change for 5–7 mV (10–12), but the addition of the polyvalent anion dextran sulfate increases the voltage dependence to as much as an *e*-fold change for 0.5 mV.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

A model is presented to account for the observed phenomena. It is proposed that significant access resistance at the mouth of VDAC channels results in voltage-dependent accumulation of polyvalent anion at the channel mouth. Electrostatic interactions between a polyvalent ion at the mouth and gating charges on VDAC would result in an augmented probability of channel closure.

EXPERIMENTAL PROCEDURES

Materials. Dextran sulfate (8 kDa and 500 kDa) and polyaspartic acid (15 kDa) were obtained from Sigma. Dextran sulfate (8 kDa) was further purified by gel filtration through a Sephadex G-25 column, lyophilized, and stored (desiccated) at room temperature. Cultures of a wall-less mutant of *Neurospora crassa* (FGSC 326) were grown as described (13) except the Nelson's medium B was supplemented with 2% (wt/vol) mannitol (Sigma)/0.75% (wt/vol) Bacto nutrient broth/0.75% (wt/vol) Bacto yeast extract (Difco).

VDAC Isolation. Mitochondrial outer membranes were isolated essentially as described by Mannella (13), supplemented with dimethyl sulfoxide to 15% (vol/vol), and stored at −70°C. Prior to experimentation, Triton X-100 [final concentration 1% (vol/vol)] was added to an aliquot and allowed to stand at room temperature for at least 30 min.

Generation of Planar Lipid Bilayers. Planar phospholipid membranes were generated by the monolayer method of Montal and Mueller (14) as described (10), using soybean phospholipids (purified as described in ref. 15). A Saran partition separated two compartments (labeled cis and trans), each containing 1 M LiCl and 5 mM $CaCl_2$. Triton X-100-solubilized outer membranes (3–5 μl) were added to the cis side of the membrane (while stirring) and VDAC was inserted spontaneously. Channel insertion could be halted by the addition of 30–50 μl of a sonicated 1% (wt/vol) dispersion of soybean phospholipid in water.

Analysis of Voltage Dependence of VDAC. The analysis of the voltage-dependent properties of VDAC follows a modification of described procedures (10, 16). A symmetrical 4-mHz triangular voltage wave (from ±10 to ±75 mV depending on the experiment) was applied to a VDAC-containing membrane, and the resulting current was recorded. The recordings were digitized and converted to conductance values. Only that part of the wave during which the electric field was decreasing with time was used for subsequent analysis.

Assuming a two-state process, the conductance–voltage curves were fitted to the Boltzmann distribution as follows:

$$\ln (G - G_{min}/G_{max} - G) = (-nFV + nFV_o)/RT. \quad [1]$$

G, G_{max}, and G_{min} are the conductance at any voltage V, the maximum conductance (all channels open), and the minimum conductance (all channels closed), respectively. F, R, and T are the Faraday constant, the gas constant, and the absolute temperature, respectively. V_o is the voltage at which one-half of the channels are closed, and n is a measure of the steepness of the voltage dependence. $(G - G_{min}/G_{max} - G)$ is equal to

the ratio of the number of open to the number of closed channels. Plots of $\ln(G - G_{min}/G_{max} - G)$ vs. V yielded n and V_o.

RESULTS

Effect of Dextran Sulfate on Voltage Dependence of VDAC. Channels were inserted into a planar phospholipid membrane bathed on both sides by 1 M LiCl/5 mM $CaCl_2$. Steps of voltage were applied to the membrane and ion permeability was monitored by measuring the current flow through the membrane. If the channels in the membrane were voltage independent, the current would increase linearly with voltage. In the case of VDAC, however, if the applied voltage is large enough, the current decreases with time as channels close. In the control recordings (Fig. 1), channel closure began at 10 mV applied potential but was not clearly evident until 15–20 mV were applied. By contrast, in the presence of 25 mM dextran sulfate (8 kDa), channel closure was evident and complete at 3 mV applied potential. Higher membrane potentials further increased the rate of closure (similarly with negative voltages). Thus, dextran sulfate dramatically increased the probability of VDAC closing, so that the channels closed completely at transmembrane voltages at which they would normally have been open.

The amplification of the voltage dependence of VDAC is a function of dextran sulfate concentration in the medium. This is illustrated in Fig. 2 by the conductance–voltage relationships. In the absence of dextran sulfate, the probability of finding VDAC in the open state decreased by a factor of e for every 6.3-mV increase in transmembrane potential. As little as 6 μM dextran sulfate increased the voltage dependence of VDAC. At 25 mM, VDAC exhibited an extremely high voltage sensitivity, an e-fold change in the probability of being open for 0.5 mV. The entire population of channels thus shifted from the open to the closed conductance state over a very narrow potential range. Note that the higher voltage dependence was accompanied by channel closure at lower voltages (Figs. 1 and 2). These changes were quantitated as n and V_o and are plotted in Fig. 3.

Effect of Dextran Sulfate on Conformational Energy of VDAC. The energy difference between the open and closed states has both voltage-independent and voltage-dependent components. The intrinsic conformational energy is the energy difference between the two states in the absence of an electric field. This can be measured by applying a potential that closes one-half of the channels. At this potential (V_o), the open and closed states are equally probable—i.e., the conformational energy difference is balanced by the application of a voltage. This energy difference is nFV_o.

The voltage-dependent energy component is nFV. Dextran sulfate increases the voltage dependence of VDAC by increasing the parameter n (Fig. 3). It produces a parallel decrease in V_o so that nFV_o remains essentially constant. The results are consistent with the conclusion that dextran sulfate simply alters the voltage dependence of VDAC without changing the total energy needed to close the channels (i.e., the conformational energy at V = 0). (For a similar treatment, see refs. 11 and 12.)

Effect of Other Polyvalent Anions. The steepening of the voltage dependence of VDAC was not caused uniquely by dextran sulfate (8 kDa). A higher molecular mass dextran sulfate (500 kDa) and a homopolymer of aspartic acid produced similar results (Table 1).

DISCUSSION

Theory. These observations have suggested a model for the effect of dextran sulfate. For a highly conductive channel like VDAC, a major impediment to ion flow through its pore may be the rate at which ions diffuse from the bulk phase to the mouth of the pore (17, 18). If access were a limiting step, flow through the channel in response to an applied potential would result in depletion of anions at one end of the channel and their accumulation at the other (conversely for cations). A local positive potential would develop in the vicinity of the channel mouth on the anion-deficient side. (Anion build-up at the opposite end of the channel would induce a local negative potential there.) Dextran sulfate should partition into this positive region where it can interact with VDAC. Furthermore, as the transmembrane potential is increased, the flow

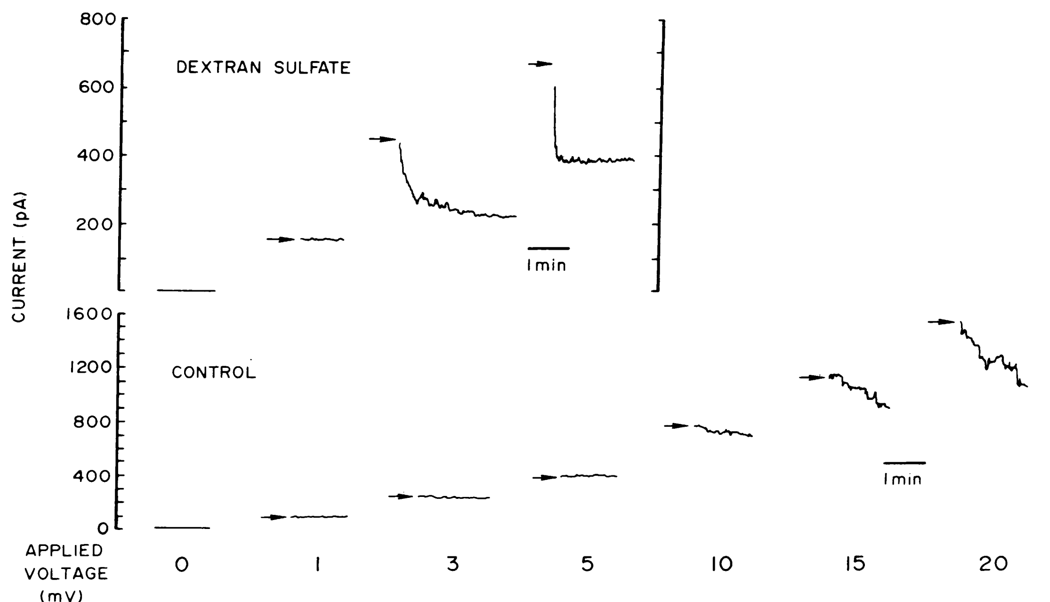

FIG. 1. Effect of dextran sulfate (8 kDa) on voltage-dependent closure of VDAC. Upper and lower traces (performed on separate membranes) show current in the presence and absence, respectively, of dextran sulfate. Applied voltages (trans minus cis) are indicated below the current traces. Arrows indicate peak instantaneous current. In the presence of dextran sulfate (25 mM), current was constant at 0 and 1 mV. Increasing the voltage to 3 mV initially increased the current ohmically. Current then decreased as the channels began to close. At 5 mV, closure was too rapid for the instantaneous current to be monitored by the chart recorder. In the absence of dextran sulfate, VDAC did not begin to close below 10 mV. Closure was still slow at 20 mV.

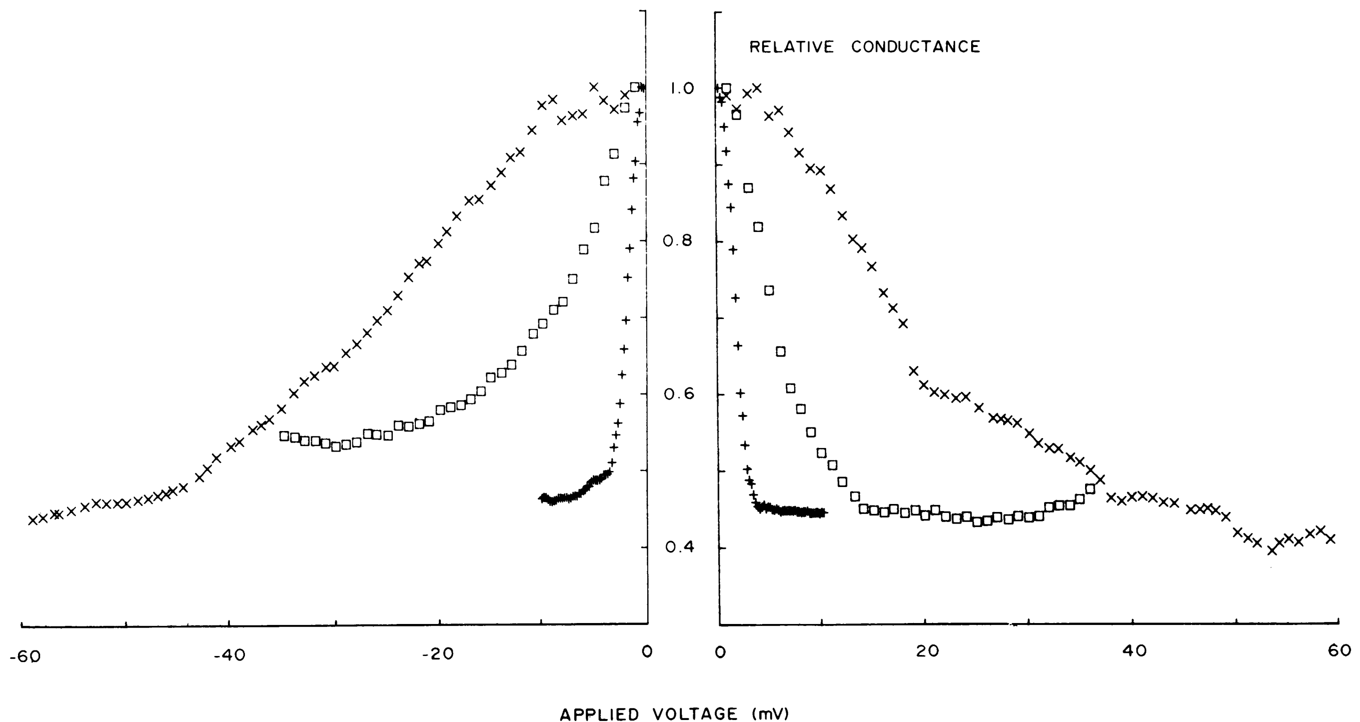

FIG. 2. Dependence of voltage sensitivity of VDAC on dextran sulfate (8 kDa) concentration. All dextran sulfate additions were symmetrical. In these experiments, the voltage was varied continuously, the current was monitored, and the conductance was calculated. Each point represents the average of four determinations. The results were then normalized to the maximum conductance. The data shown were collected in the absence of dextran sulfate (×) and in the presence of dextran sulfate at 6.25 μM (□) and 25 mM (+). The voltages were changed at rates of 1.2, 0.56, and 0.16 mV/sec, respectively. The respective voltage dependences are *e*-fold for 6.3, 2.0, and 0.5 mV. Voltages are cis relative to trans.

of ions through the channels would increase, resulting in a greater depletion of anions at one end of the pore and, consequently, a larger local positive potential and more dextran sulfate partitioning. Thus, the build-up of dextran sulfate near the membrane would itself be voltage dependent.

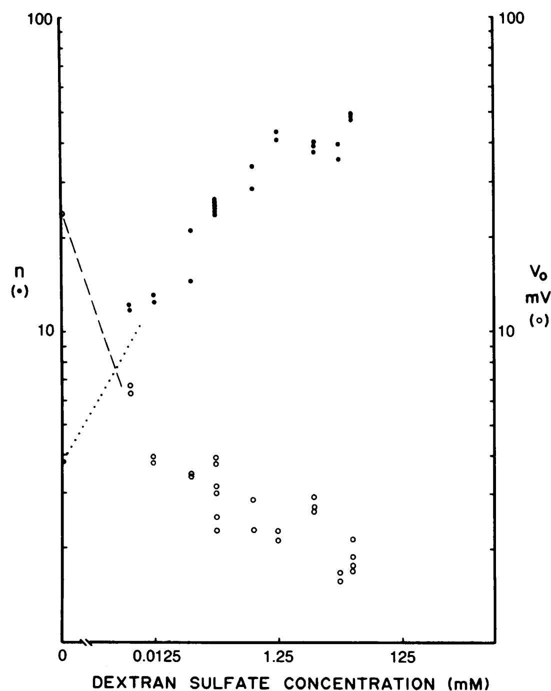

FIG. 3. The parameters n and V_o as a function of dextran sulfate (8 kDa) concentration. Each symbol represents an average determination made from three or four triangular wave applications to a VDAC-containing membrane.

A highly schematic representation of the potential and dextran sulfate concentration profiles postulated by this theory are presented in Fig. 4 (*Inset*).

Quantitation. In the absence of dextran sulfate, the probability of a channel being in the open or closed state is a function of the balance between the intrinsic conformational energy and the energy provided by an applied potential:

$$RT \ln(\text{closed/open}) = nF(1 - 2r)\, V - \Delta G, \quad [2]$$

where r is the fraction of the potential falling in the bulk phase; $(1 - 2r)$ corrects for that portion of the applied potential not felt by the channel; ΔG is the conformational energy difference between the open and closed states; open and closed are the probabilities of finding a channel in the open or closed conformation, respectively; and n, F, R, and T are the same as for Eq. 1.

In the presence of dextran sulfate, an additional energy term is added:

$$RT \ln(\text{closed/open}) = nF(1 - 2r)V - \Delta G + mP_d, \quad [3]$$

where P_d is the probability of finding a dextran sulfate

Table 1. Gating properties of VDAC in the presence of various polyanions

Agent	Concentration, μM	(mg/ml)	n	V_o
Dextran sulfate (8 kDa)	6	(0.05)	10.0	5.5
	62	(0.5)	14.7	3.2
	125	(1)	25.4	3.5
	625	(5)	31.7	2.5
Dextran sulfate (500 kDa)	2	(1)	52.9	2.7
	10	(5)	64.7	1.3
Polyaspartic acid (15 kDa)	67	(1)	22.1	4.3

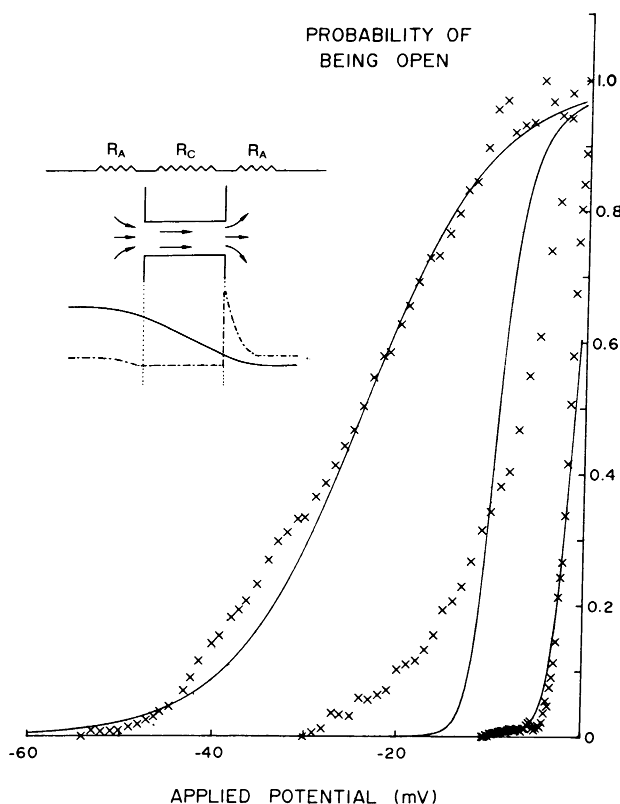

FIG. 4. Model of the ultrasteep voltage dependence. (*Inset*) Simple representation of the effect of access resistance (R_A) on voltage (V) (——) and dextran sulfate concentration (– – – –) profiles in the bulk phase and adjacent to the membrane in the vicinity of VDAC. Anion depletion and cation accumulation on the low-potential side induces a local positive region adjacent to the membrane, relative to the bulk solution, into which dextran sulfate partitions. R_C, channel resistance. The main figure shows probability of VDAC being in the open state as determined from experiments (×) and as predicted from Eq. 3 (curves). Dextran sulfate (8 kDa) concentrations were 0, 6 μM, and 12.5 mM. Experimental probabilities were generated from the corresponding conductance–voltage relationships by setting the minimum conductance equal to zero probability.

molecule at the mouth of the pore,* and m is a measure of the electrostatic interaction between dextran sulfate and the gating charges in VDAC. A reasonable expression for P_d might be

$$P_d = \frac{D^i \exp(zFrV/RT)}{K + D^i \exp(zFrV/RT)} \quad [4]$$

where D is the dextran sulfate concentration in the bulk phase, i is the power dependence of the dextran sulfate concentration, z is the valence of dextran sulfate (≈50), and K is the effective concentration of dextran sulfate at the mouth when P_d is equal to 1. Fig. 4 shows the fit between open probabilities obtained experimentally in the presence of dextran sulfate and those predicted by Eq. 3. Allowing m, r, i, and K to vary within reasonable ranges, lines were fitted to the data for 0, 6 μM, and 12.5 mM. The fitted values were as follows: m = 25,000 J, r = 0.15, i = 0.4, and K = 0.5 M. [When calculated from Coulomb's law, the pure electrostatic attraction between dextran sulfate and the gating charges of VDAC (assuming a reasonable separation distance) yields an m value of 2–3 × 10^6 J. This energy is an overestimation because the calculation assumes point charges separated by a uniform dielectric. The charge distribution and salt screening in 1 M LiCl should greatly reduce the estimate.]

Predictions. This model makes several predictions, some of which have been tested. Only those tests whose results are complete are presented.

The theory stipulates that the effect on the voltage dependence of VDAC is not dependent on a specific binding interaction between VDAC and dextran sulfate. Rather, it is the polyanionic nature of the molecule that is important for increasing the voltage dependence of the channel. Similar results should be obtained with other polyvalent anions. This is the case. Table 1 shows that 67 μM polyaspartic acid increased the voltage dependence of VDAC ≈6-fold while V_o decreased. This is comparable to the results obtained using 62 μM dextran sulfate.

Second, in accordance with the Boltzmann relation, the extent to which a polyvalent anion partitions into the local positive region near the channel's mouth depends on its charge. Consequently, the greater the charge on the ion, the larger should be the apparent increase in the voltage dependence of VDAC. Indeed, 67 μM polyaspartic acid (15 kDa) steepens the voltage dependence of VDAC to a significantly greater extent than 62 μM dextran sulfate (8 kDa). Assuming 90% dissociation at pH 7.0 (20), polyaspartic acid has a valence of 115, while that of dextran sulfate is only 50. Dextran sulfate (500 kDa) also caused a much greater steepening in voltage sensitivity of VDAC than a similar concentration of dextran sulfate (8 kDa) (see Table 1).

Third, the local positive region into which polyvalent ions partition is due to anion depletion at the negative potential end of the channel. Therefore, polyvalent anions should increase the voltage dependence of VDAC only when present on the negative side of the membrane. Fig. 5 shows the results of a representative experiment in which dextran sulfate (125 μM) was added to one side (the cis side) of a VDAC-containing membrane. When this side was made negative (which should result in dextran sulfate accumulation at the mouth of the pore), VDAC exhibited ultrasteep voltage dependence. The channels showed a tendency to close at −2.6 mV and closed completely at −4.6 mV. Positive voltages of similar magnitudes applied to the cis side (which should result in dextran sulfate depletion) produced no closure. This observation is consistent with the proposed model.

The model presented here correlates with the finding that positive charges, probably lysine ε-amino groups, are responsible for gating in VDAC (11, 12, 21). A polyvalent anion, accumulating near the channel mouth in a voltage-dependent manner, could induce closure via electrostatic interactions with the gating charges. Once closed, the channel would be stabilized in that state by the proximity of the polyvalent anion (recall that closed VDAC channels still conduct ions but at a slower rate, so that the access problem may still exist). These findings are also consistent with a recent report that, in the closed state of the channel, VDAC's gating charges are more accessible to the negative side of the membrane (21). This is the same side of the membrane where the polyvalent anions are effective.

These results represent a unique way to control voltage-gated channels. Channels could be opened and excitable phenomena initiated by altering the effective concentration of a polyvalent ion. This could occur as a result of protein phosphorylation, the release of Ca^{2+}, which could bind to and reduce the charge on a polyvalent anion, pH changes, etc. Whether this phenomenon

*Since dextran sulfate (8 kDa) is too large to enter VDAC's pore (19) and could be expected to fully occupy the space immediately adjacent to the channel mouth, it is more appropriate to use this probability than the dextran sulfate concentration next to the membrane when considering the extent of the electrostatic effects of dextran sulfate on the gating behavior of individual channels. This results in a function that saturates when the probability is 1. The dextran sulfate concentration profile of Fig. 4 (*Inset*) is equally representative of this probability.

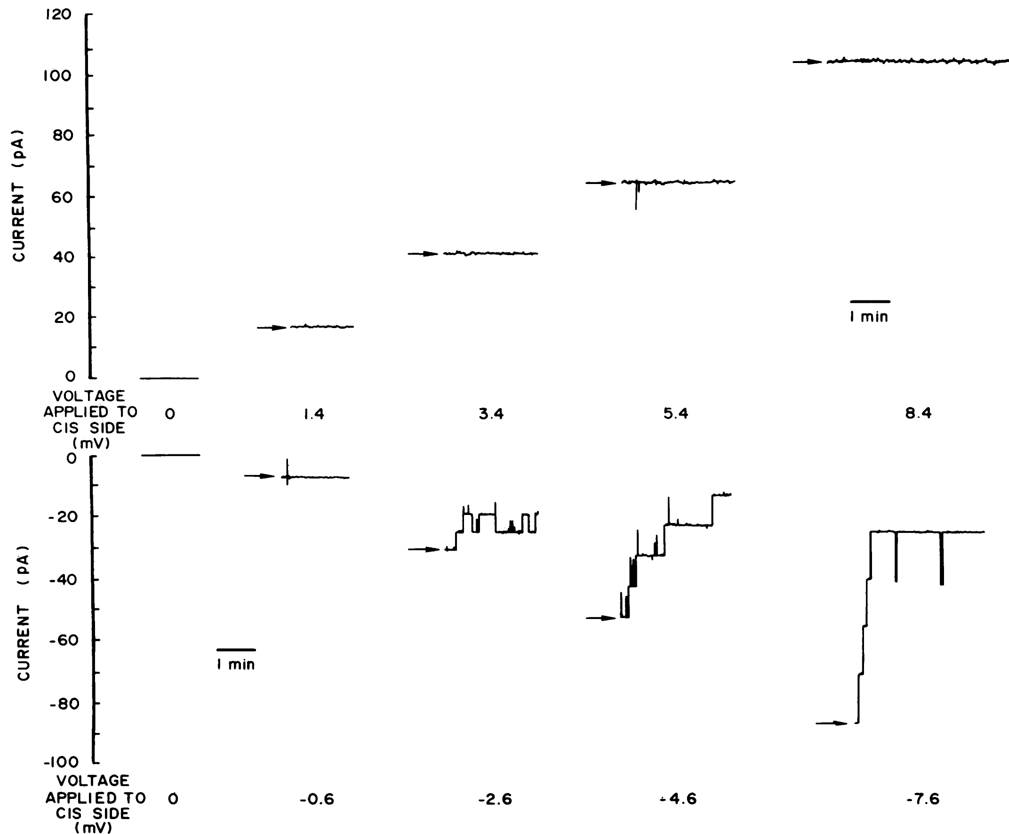

FIG. 5. Asymmetry of effect of dextran sulfate (8 kDa) on voltage dependence of VDAC. VDAC was incorporated after dextran sulfate was added to the cis side (final concentration, 125 μM). Upper traces show current responses to positive voltages applied to the dextran sulfate-containing side of the membrane. In the lower traces, voltages of similar magnitudes but of opposite sign were applied to the same membrane. Arrows indicate instantaneous current levels prior to channel closure.

is generally applicable or has any physiological relevance to VDAC or any other channel has yet to be determined. However, in the case of VDAC, it is hard to imagine why the voltage-dependent properties of VDAC have been so highly conserved if not to regulate molecular traffic across the outer mitochondrial membrane. The mechanism presented here is a plausible method of control.

Two conditions are necessary if this model is to have significance for the function of VDAC: polyvalent anion(s) and a potential across the outer mitochondrial membrane. Magnesium-binding proteins in the intermembrane space may be candidates for *in vivo* counterparts of dextran sulfate (22). The small amount of anion necessary to induce a shift in voltage dependence is biologically feasible. As little as 6.25 μM dextran sulfate increased the voltage sensitivity of VDAC 3-fold. The transmembrane voltage needed to accumulate polyvalent anions at the channel mouth need not be large and could be generated by a Donnan potential.

The effects of polyvalent (di- and trivalent) metal ions on Na^+ and K^+ channels have been described (23, 24). However, unlike the effect we describe, those effects did not result in an increase in the steepness of the voltage dependence and were consistent with alterations in the membrane surface potential. We encourage others to look for similar effects in other voltage-gated channels.

This work was supported by Office of Naval Research Grant N000014-85-K-0651 and by Biomedical Research Support Grant RR-07042 to the University of Maryland from the Division of Research Resources, National Institutes of Health.

1. Hodgkin, A. L. & Huxley, A. F. (1952) *J. Physiol. (London)* **117**, 500–544.
2. Parsons, D. F., Bonner, W. D., Jr., & Verboon, J. G. (1965) *Can. J. Bot.* **43**, 647–655.
3. Colombini, M. (1979) *Nature (London)* **279**, 643–645.
4. Zalman, L. S., Nikaido, H. & Kagawa, Y. (1980) *J. Biol. Chem.* **255**, 1771–1774.
5. Freitag, H., Neupert, W. & Benz, R. (1982) *Eur. J. Biochem.* **123**, 629–636.
6. Roos, N., Benz, R. & Brdiczka, D. (1982) *Biochim. Biophys. Acta* **686**, 204–214.
7. Linden, M., Gellerfors, P. & Nelson, B. D. (1982) *Biochem. J.* **208**, 77–82.
8. Mannella, C. A. & Colombini, M. (1984) *Biochim. Biophys. Acta* **774**, 206–214.
9. Mannella, C. A., Radermacher, M. & Frank, J. (1984) *Proc. Annu. Electron Microscopy Soc. Am. Meet.* **42**, 644–645.
10. Schein, S. J., Colombini, M. & Finkelstein, A. (1976) *J. Membr. Biol.* **30**, 99–120.
11. Doring, C. & Colombini, M. (1969) *J. Membr. Biol.* **83**, 81–86.
12. Bowen, K. A., Tam, K. & Colombini, M. (1985) *J. Membr. Biol.* **86**, 51–60.
13. Mannella, C. (1982) *J. Cell Biol.* **94**, 680–687.
14. Montal, M. & Mueller, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3561–3566.
15. Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem.* **246**, 5477–5487.
16. Ehrenstein, G., Lecar, H. & Nossal, R. (1970) *J. Gen. Physiol.* **55**, 119–133.
17. Lauger, P. (1976) *Biochim. Biophys. Acta* **455**, 493–509.
18. Anderson, O. S. (1983) *Biophys. J.* **41**, 147–165.
19. Colombini, M. (1980) *Ann. N.Y. Acad. Sci.* **341**, 552–563.
20. Berger, A. & Katchalski, E. (1951) *J. Am. Chem. Soc.* **73**, 4084–4088.
21. Doring, C. & Colombini, M. (1985) *J. Membr. Biol.* **83**, 87–94.
22. Bogucka, K. & Wojtczak, J. (1976) *Biochem. Biophys. Res. Commun.* **71**, 161–167.
23. Frankenhaeuser, B. & Hodgkin, A. (1957) *J. Physiol. (London)* **137**, 218–244.
24. Starzak, M. & Starzak, R. (1978) *Biophys. J.* **24**, 555–560.