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Voltage Dependence and Ion Selectivity of the Mitochondrial Channel, VDAC, are Modified by Succinic Anhydride

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Summary. The mitochondrial protein VDAC forms voltage-dependent anion-selective channels in planar phospholipid membranes. When succinic anhydride was added to these membranes, it virtually eliminated VDAC's voltage-dependence and changed its selectivity from anion to cation. These results were obtained without large changes in open-channel conductance or in energy difference between the open and closed states in the absence of a field. Since succinic anhydride converts amino groups into carboxyl groups, we propose that amino groups are responsible for VDAC's voltage-dependence and anion selectivity. Perhaps the same charges are responsible for both functions.

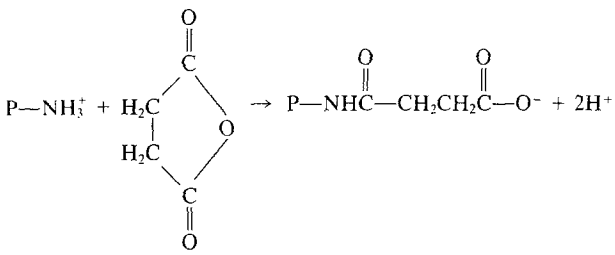
Key Words voltage-gating · outer membrane · membrane · ion transport · protein modification · planar bilayer membrane

Introduction

VDAC (voltage dependent anion-selective channel) is a protein located in the outer membrane of mitochondria (Parsons, Williams & Chance, 1966; Mannella & Bonner, 1975; Schein, Colombini & Finkelstein, 1976; Colombini, 1979; Zalman, Nikaido & Kagawa, 1980; Freitag, Neupert & Benz, 1982; Linden, Gellerfors & Nelson, 1982; Roos, Benz & Brdiczka, 1982; Manella & Colombini, 1984). It forms large water-filled channels (Colombini, 1980*b*; Zalman et al., 1980) in these membranes thus allowing small molecules to travel between the cytoplasm and the mitochondrial intramembrane space. An electric field will cause these channels to assume a less conductive conformation known as the closed state. This voltage-dependence is very pronounced (a small change in voltage can result in a dramatic change in conductance) and highly conserved among all mitochondria tested (Colombini, 1979; 1980*b*).

The molecular basis for the voltage-control of channel-forming proteins is not understood. Although the mechanism underlying the voltage-dependence of some channel-forming ionophores is understood fairly well (Eisenberg, Hall & Mead, 1973; Heyer, Muller & Finkelstein, 1976), this mechanism probably does not apply to the channel-forming proteins. Theoretically, one way of achieving voltage-dependence is to use charges on the protein to sense the electric field. The presence of these charges might make a particular conformational state of the protein energetically more stable in the presence of an electric field than in the absence of the field. Thus the probability of finding the protein in this conformational state will increase with increasing magnitude of the electric field. If this state happened to be less conductive than the preferred state in the absence of the field, then the membrane conductance would decrease with increasing electric field.

In this paper we have used the amino group modifier (Habeeb, Cassidy & Singer, 1958), succinic anhydride, to determine if amino groups might be involved in the voltage-dependent mechanism. This anhydride reacts with amino groups as follows:



An amino group is essentially converted into a carboxyl group so that, at physiological pH, the net positive charge becomes a net negative charge. This reagent alters both the selectivity and voltage-dependence of VDAC channels. Some of these results have been published in abstract form (Doring & Colombini, 1983).

Table 1. Succinic anhydride modifies VDAC's ion selectivity

Medium[a]	Succinic anhydride added (μmol)	Reversal potential (mV)	Permeability ratio (anion/cation)	
			U^-/U_+[b]	P^-/P_+[c]
1 M KCl *vs.*	0	+10	1.5	1.7
0.1 M KCl	8	−11	0.63	0.55
	17	−19	0.43	0.35
	25	−23	0.35	0.25
	33	−25	0.31	0.24
1 M LiCl *vs.*	0	+20	2.2	2.8
0.1 M LiCl	12	+ 3	1.1	1.2
	25	−11	0.65	0.57
	50	−18	0.49	0.39
	75	−20	0.45	0.35

[a] The KCl medium was buffered with 50 mM $NaHCO_3$ while the LiCl medium was buffered with 50 mM MOPS, pH 7.5.
[b] Nernst-Planck treatment.
[c] Goldman-Hodgkin-Katz treatment.

Materials and Methods

Planar phospholipid membranes, consisting of soybean phospholipids, were generated by the monolayer method of Montal and Mueller (1972) and as previously described (Schein et al., 1976). VDAC was purified from rat liver according to the method of Colombini (1983) and fraction 1 was used routinely. This consisted of partially purified VDAC in 0.7% Triton X-100, 20% DMSO, 0.5 M KCl, 0.05% Na^+ azide, 1 mM HEPES (Na^+ salt, pH 7.0). A small aliquot of this (usually 10 μl) was added to the aqueous phase on one side (labeled the *cis* side) of the membrane. VDAC inserts spontaneously into the membrane (Colombini, 1979). If it was desirable to limit the number of channels in the membrane (e.g. to perform experiments on a single channel), 50 μl of a 10 mg/ml solution of Concanavalin A was added to the *cis* side. (Con A inhibits VDAC insertion into planar phospholipid membranes.) All experiments were done under voltage-clamp conditions. Succinic anhydride was added to the aqueous phase from a solution of the anhydride in dimethylsulfoxide (83 or 166 mg/ml).

The reagent, succinic anhydride, is a small molecule relative to the size of VDAC's pore (Colombini, 1980*a*) and should therefore be able to diffuse through VDAC to the other side of the membrane [Succinate can easily cross a membrane via VDAC, (Colombini, 1980*a*).] In spite of this, the side of the membrane to which succinic anhydride was added may determine the type and degree of modification because of the anhydride's short half-life in water (about 2 min). Indeed the accompanying paper (and also Doring & Colombini, 1984) reports that modification may depend on which side of the membrane the anhydride was added. However, when modification was performed on channels in the open state, the side to which anhydride was added did not appear to make any difference. In the experiments reported in this paper, most of the additions were made to the *cis* side but some were made to the *trans* side.

The permeability ratios (anion permeability/cation permeability) reported in Table 1 were calculated either according to the Nernst-Planck treatment or by means of the Goldman-Hodgkin-Katz equation. In addition to the assumptions inherent in these treatments, we assumed that the cations and anions of the added buffer and the succinate generated by the reaction behave exactly like the cations and anions of the salt. This introduces only a small error and in no way alters the conclusion that anhydride reaction drastically changes VDAC's ion selectivity.

The analysis of the voltage-dependence of the channels prior to modification was performed by methods very similar to those described previously [Ehrenstein, Lecar and Nossal (1970) for EIM and Schein et al. (1976) for VDAC]. A symmetrical 5-mHz triangular voltage wave (from ±60 to ±80 depending on the experiment) was applied to the VDAC-containing bilayer membrane. The resulting current was recorded but only part of the wave (that part in which the electric field was decreasing with time) was used in the subsequent analysis. The mode of analysis which we used assumed that each channel can exist in one of two states, open or closed. Although we know that VDAC channels from rat liver can close to many different closed states (Colombini, 1980), we decided to make this assumption for the following reasons: 1) the results of the analysis were consistent with a two-state system in that a straight line was obtained when the data were fitted to the equation (1) (a curve would have resulted if the voltage dependence of the energy difference between the open state and each of the closed states was very different); 2) since for each channel there exists a whole spectrum of closed states with different conductance levels (the closed states are not completely closed), a mathematical analysis of such a system would be extremely difficult; 3) although one can question the exact meaning of the values of the parameters used to describe the voltage-dependence, there is no question that these values are a valid measure of the steepness of the voltage-dependence irrespective of the number of closed states. Measurements of current as a function of voltage were digitized, converted to conductance and fitted to the following equation (essentially Eq. (2) from Schein et al., 1976):

$$\ln\left(\frac{G - G_{\min}}{G_{\max} - G}\right) = \frac{-nFV + nFV_o}{RT}. \tag{1}$$

G, $G_{\max}$, and $G_{\min}$ are the conductance at any voltage V, the maximum conductance (when all the channels are open), and the minimum conductance (when all channels are 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

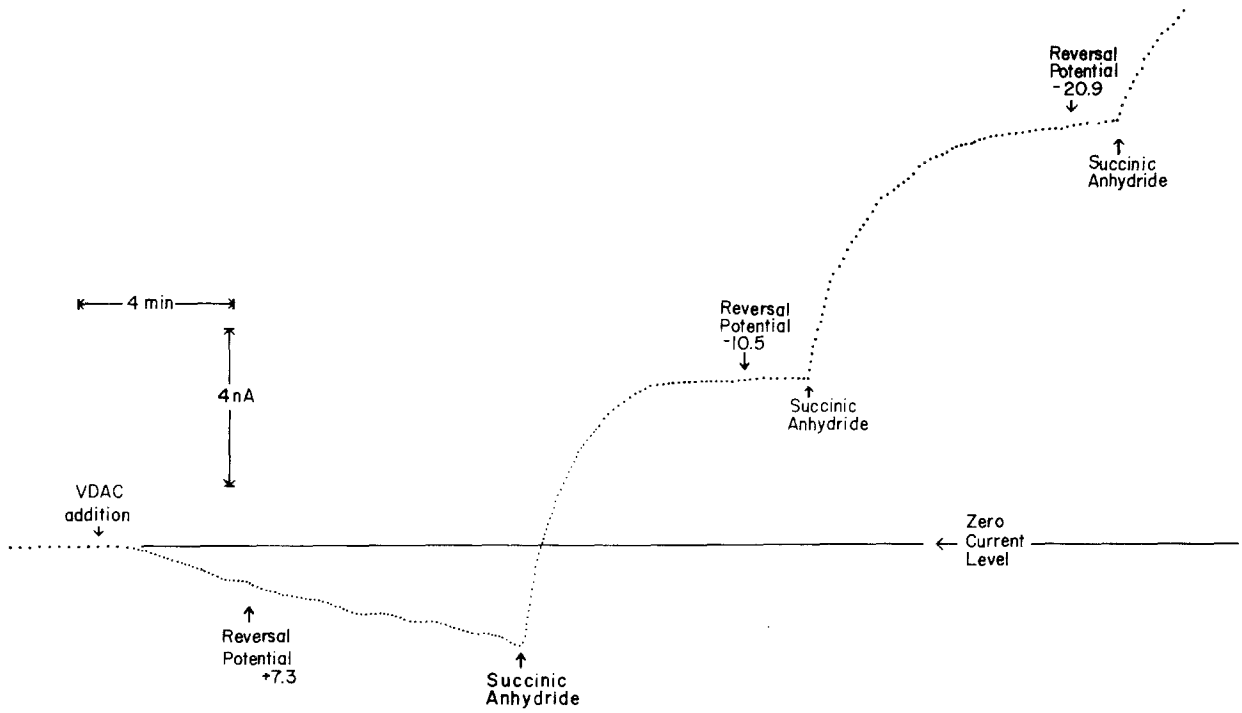


Fig. 1. Succinic anhydride modified VDAC's ion selectivity. At the point indicated in the Figure, 10 μl of a Triton X-100 solubilized VDAC fraction were added to the *cis* side of a planar phospholipid bilayer membrane (1.0 M KCl, 50 mM Na MOPS, pH 7.5, on the *cis* side and 0.1 M KCl, 50 mM Na MOPS, pH 7.5, on the *trans* side). The current (positive current was defined as positive charge traveling from the *cis* to the *trans* side) was monitored with the voltage clamped at zero. At the points indicated, the voltage needed to bring the current to zero instantaneously (the reversal potential) was measured. Eight micromoles of succinic anhydride were added to the *cis* side at the times indicated

half the channels are closed and n is a measure of the steepness of the voltage dependence. When half the channels are closed, the energy level of the open state should be equal to that of the closed state(s). Therefore nFV_o is the energy which must be applied to compensate for the difference in conformational energy between the states in the absence of an applied field. nFV_o is therefore a measure of this energy difference.

The analysis of the modified channels presented one major difficulty. Associated with modification, the value of n decreased and the value of V_o increased. As a result it became difficult to obtain a value for G_{min} to use in the above equation. From single-channel experiments it was clear that although the single-channel conductance decreased with modification, the percentage drop in conductance upon voltage-dependent closure did not change appreciably upon modification (as performed in these experiments). Hence, for the modified channels, the value for G_{min} was calculated from the G_{max} value using the percentage closure figure determined prior to modification.

The reagents used in these experiments were purchased from Sigma Chemical Co., St. Louis, Mo.

Results

Succinic Anhydride Reverses VDAC's Ion Selectivity

These experiments were performed in the presence of a tenfold concentration gradient across the membrane. A typical experiment is shown in Fig. 1. At the time indicated an aliquot of VDAC-detergent solution was added to the aqueous phase bathing the *cis* side of the phospholipid bilayer membrane (*see* Materials and Methods). No voltage was applied since VDAC's preference for anions resulted in a current flow. At various times during the experiment the reversal potential (i.e. the voltage needed to bring the current to zero) was measured. This was used to estimate the degree of ion selectivity using either the electroneutrality assumption (Nernst-Planck) or the constant field assumption (Goldman-Hodgkin-Katz). Succinic anhydride was added as indicated in the Figure resulting in a dramatic change in ion selectivity. The measured current changes direction reflecting a reversal of the normal ion selectivity. Table 1 shows the results of typical experiments in KCl and LiCl media. The sequential addition of anhydride caused progressively more change in ion selectivity. Addition of dimethylsulfoxide alone had no effect (*data not shown*). The ratio of the permeability of the anion to the cation changes from five- to 10-fold depending on the experiment, the ion composition of the medium, and the theoretical treatment used to perform the analysis. In all cases, the channel's normal pref-

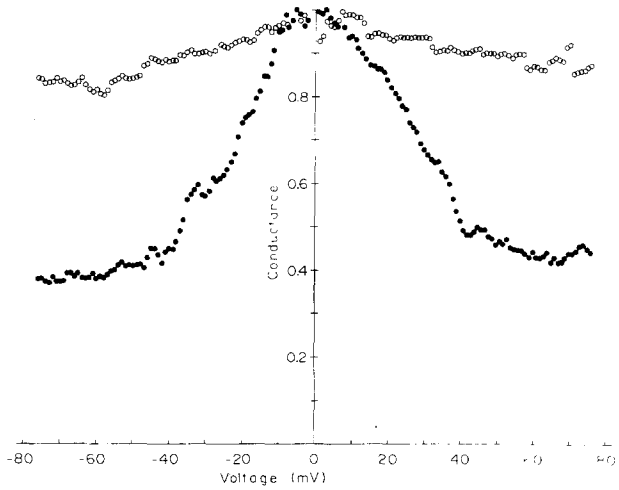


Fig. 2. Succinic anhydride modified VDAC's voltage dependence. These experiments were performed in symmetrical solutions: 1.0 M LiCl, 5 mM $CaCl_2$, 50 mM LiMOPS, pH 7.2. The data shown were obtained from the same VDAC-containing membrane before (●) and after (○) the addition of 8 μmol of succinic anhydride to each side of the membrane

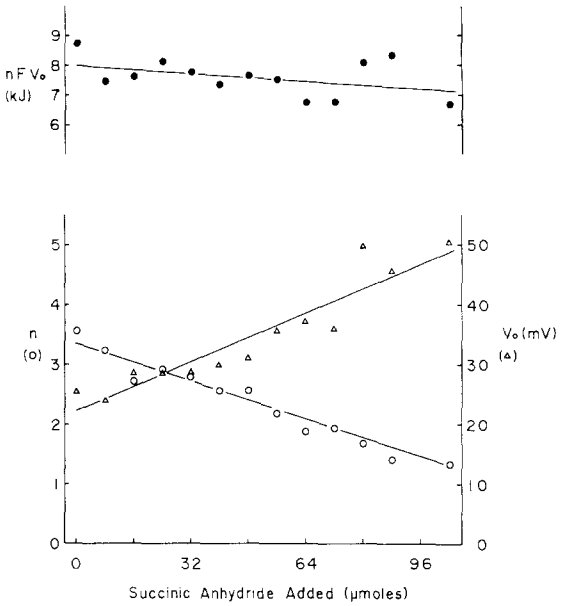


Fig. 3. The steepness of VDAC's voltage-dependence was reduced in a dose-dependent manner. The values of n (○), V_o (△) and nFV_o (●) were calculated as described in Materials and Methods for data obtained as in Fig. 2 (except that the aqueous medium contained KCl instead of LiCl). The points are averages of 2 to 6 estimations and were all obtained on one VDAC-containing membrane. The lines drawn through the data were calculated by the method of least squares

erence for anions was converted into a preference for cations.

Succinic Anhydride Inhibits VDAC's Voltage-Dependence

These experiments were performed with equal concentrations of salts on the *cis* and *trans* sides of the membranes. Voltage dependence was quantitated by applying a triangular voltage wave and measuring the resulting current. Since VDAC's kinetics of opening are fast (Schein et al., 1976; Colombini, 1979), that part of the response in which channels were opening (decreasing electric field) was used to calculate the conductance as a function of applied voltage. Figure 2 shows how conductance varies with voltage prior to and after anhydride modification. The obvious loss in voltage-dependent conductance was quantitated (*see* Materials and Methods) by determining the steepness of the voltage-dependent change in conductance n (the number of charges which would have to traverse the entire voltage difference in order to account for the steepness of the voltage dependence) and the voltage at which half the channels are open V_o. Figures 3 and 4 show how these parameters change with incremental additions of anhydride for experiments in KCl and LiCl media. n decreases with modification while V_o increases. The energy difference between the open and closed state in the absence of a field nFV_o does not change very much with modification. Linear regression of the KCl data (Fig. 3) yielded correlation coefficients of -0.97, $+0.93$ and -0.43 for the n, V_o and nFV_o values, respectively.

The most straightforward interpretation of the results just described is that anhydride modification results in an increase in the probability of finding VDAC in the open state. This was tested directly by modifying a single channel (Fig. 5). The probability of the channel being open at a given voltage was obtained by analyzing records of current response to a triangular voltage wave. The results shown in Fig. 5 were obtained with one channel on one membrane. Succinic anhydride modification reduced this channel's voltage-dependence by increasing the probability of finding the channel in the open state. When these curves were analyzed as described above for multi-channel membranes, the results shown in Table 2 were obtained. As was found with the multi-channel membranes, the value of n dropped while that of V_o increased and that of nFV_o remained relatively unchanged.

Discussion

One way in which a channel-forming protein can respond to an applied electric field is to have a set of

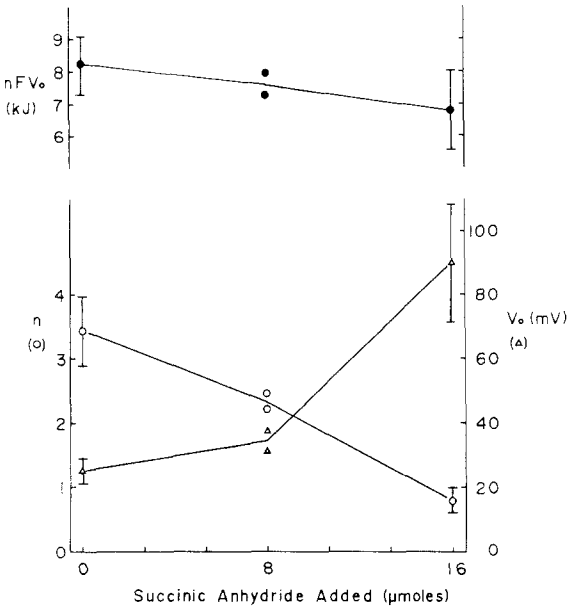


Fig. 4. Steep reduction of VDAC's voltage-dependence after modification in LiCl medium. The values of n (○), V_o (△) and nFV_o (●) were calculated as described in Materials and Methods for data obtained as in Fig. 2. The vertical lines at zero and 16 μmol of added anhydride represent the standard deviation of 9 and 4 estimates, respectively. The lines drawn in this Figure connect the means of the estimates for a given addition of anhydride

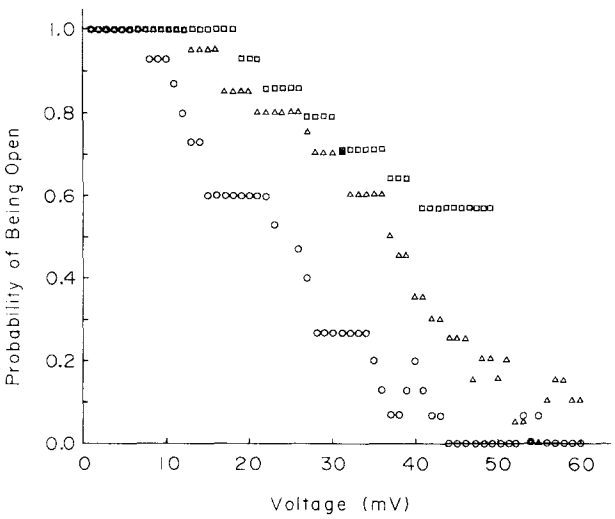


Fig. 5. Modification of the voltage-dependent properties of a single channel. The probability of finding a single channel open is plotted as a function of voltage for: 1) an unmodified channel (○), 2) the same channel after the addition of 7.5 μmol of succinic anhydride (△), and 3) the same channel after a second addition of 7.5 μmol of anhydride (□). A KCl-containing solution was used for this experiment (as in Fig. 3). The single-channel's voltage dependence was analyzed by means of a triangular voltage wave as was done for multi-channel experiments. The probability of finding the channel in the open state at a given voltage was determined by calculating the fraction of the records in which the channel was open at that voltage. The voltage-dependence parameters were calculated from the data in this Figure and are summarized in Table 2

charges which detect the electric field and as a result cause a particular conformational state of the protein to become energetically more stable in the presence of the field. These charges are normally referred to as gating charges. Since VDAC channels close with increasing electric field, the closed state becomes energetically more favored as the field is increased. According to this mechanism, if the charges were neutralized, the electric field should no longer favor the closed state of VDAC and the channel should remain open.

We have found that succinic anhydride modification results in the channels remaining open. Since amino groups are prime targets for anhydride modification, they are good candidates for the groups being modified. Reaction with succinic anhydride should convert the positive charges into negative charges. (If the charges were in close proximity, one anhydride molecule might neutralize two positive charges by reacting with both of them.) One interpretation of these results is that some of the gating charges were neutralized thus reducing VDAC's voltage dependence. The measured decrease in the parameter n is consistent with this interpretation. A reduced n means that fewer charges are needed to account for the observed voltage dependence.

Table 2. Results of modifying a single channel[a]

Succinic anhydride added (μmol)	n	V_o (mV)	nFV_o (kJ)
0	3.8	24	9.0
8	3.0	36	10.5
17	2.1	47	9.3

[a] Results of calculations performed on the data shown in Fig. 5.

Although it is possible that the observed changes in n were due to gross structural changes induced by anhydride modification, there are reasons to think otherwise. The conductance of the open channel does not change very much. The energy difference between the open and closed state in the absence of a field nFV_o also changes by only a small amount. Therefore the modifications induced by succinic anhydride are probably rather subtle. Succinylation of another channel-forming protein, porin (Hiroko, Tokunaga & Nakae, 1981), resulted in only minor structural changes as monitored by circular dichroism.

Anhydride modification also results in a dramatic change in the selectivity of VDAC. The channels go from being anion-selective to being cation-selective. This change is also consistent with the modification of amino groups. The fact that selectivity modification occurs in parallel with the modification of voltage-dependence, raises the possibility that one set of amino groups is responsible for both the channel's selectivity and its voltage-dependence.

Although VDAC's selectivity for anions is weak, it is difficult to see how any selectivity could exist in view of its large pore size (40 Å in diameter; Colombini, 1980*b*) except for selectivity induced by fixed positive charges within the pore. Therefore, the molecular basis for selectivity in large channels may be rather different from what one finds in narrow channels. For narrow channels the selectivity filter and the gating mechanism may be different structures. However, for large channels, a single structure performing both functions is easy to envisage. The accompanying paper proposes a mechanism in which this occurs.

References

Colombini, M. 1979. A candidate for the permeability pathway of the outer mitochondrial membrane. *Nature (London)* **279:**643–645

Colombini, M. 1980*a*. Structure and mode of action of a voltage-dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane. *Ann. N.Y. Acad. Sci.* **341:**552–563

Colombini, M. 1980*b*. Pore size and properties of channels from mitochondria isolated from *Neurospora crassa*. *J. Membrane Biol.* **53:**79–84

Colombini, M. 1983. Purification of VDAC (voltage-dependent anion-selective channel) from rat liver mitochondria. *J. Membrane Biol.* **74:**115–121

Doring, C., Colombini, M. 1983. Simultaneous alteration of ion selectivity and voltage dependence by reacting the channel former, VDAC, with succinic anhydride. *Biophys. J.* **41:**48a

Doring, C., Colombini, M. 1984. On the nature of the molecular mechanism underlying the voltage dependence of the channel-forming protein, VDAC. *Biophys. J.* **45:**44–46

Ehrenstein, G., Lecar, H., Nossal, R. 1970. The nature of the negative resistance in bimolecular lipid membranes containing excitability-inducing material. *J. Gen. Physiol.* **55:**119–133

Eisenberg, M., Hall, J.E., Mead, C.A. 1973. The nature of the voltage-dependent conductance induced by alamethicin in black lipid membranes. *J. Membrane Biol.* **14:**143–176

Freitag, H., Neupert, W., Benz, R. 1982. Purification and characterization of a pore protein of the outer mitochondrial membrane from *Neurospora crassa*. *Eur. J. Biochem.* **123:**629–636

Habeeb, A.F.S.A., Cassidy, H.G., Singer, S.J. 1958. Molecular structural effects produced in proteins by reaction with succinic anhydride. *Biochim. Biophys. Acta* **29:**587–593

Heyer, E.J., Muller, R.U., Finkelstein, A. 1976. Inactivation of monazomycin-induced voltage-dependent conductance in thin lipid membranes. *J. Gen. Physiol.* **67:**731–748

Linden, M., Gellerfors, P., Nelson, B.D. 1982. Purification of a protein having pore forming activity from the rat liver mitochondrial outer membrane. *Biochem. J.* **208:**77–82

Mannella, C.A., Bonner, W.D., Jr. 1975. X-ray diffraction from oriented outer mitochondrial membranes. *Biochim. Biophys. Acta* **413:**226–233

Mannella, C.A., Colombini, M. 1984. Evidence that the crystalline arrays in the outer membrane of *Neurospora* mitochondria are composed of the channel protein, VDAC. *Biochim. Biophys. Acta* **774:**206–214

Montal, M., Mueller, P. 1972. Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA* **69:**3561–3566

Parsons, D.F., Williams, G.R., Chance, B. 1966. Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Ann. N.Y. Acad. Sci.* **137:**643–666

Roos, N., Benz, R., Brdiczka, D. 1982. Identification and characterization of the pore-forming protein in the outer membrane of rat liver mitochondria. *Biochim. Biophys. Acta* **686:**204–214

Schein, S.J., Colombini, M., Finkelstein, A. 1976. Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from *Paramecium* mitochondria. *J. Membrane Biol.* **30:**99–120

Tokunaga, H., Tokunaga, M., Nakae, T. 1981. Permeability properties of chemically purified porin trimers from *Escherichia coli* B. *J. Biol. Chem.* **256:**8024–8029

Zalman, L.S., Nikaido, H., Kagawa, Y. 1980. Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels. *J. Biol. Chem.* **255:**1771–1774

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