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The Mitochondrial Voltage-Dependent Channel, VDAC, is Modified Asymmetrically by Succinic Anhydride

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Summary. In the accompanying paper, succinic anhydride was shown to react with the outer mitochondrial membrane channel-forming protein, VDAC, resulting in the loss of its voltage dependence. In this paper, the anhydride was added to VDAC held in a particular conformational state by means of an applied electric field. VDAC was inserted into the membranes from the *cis* side and the anhydride was added either to the *cis* or *trans* side. Channels modified in the open state behaved similarly whether anhydride was added to the *cis* or *trans* side. Modifications of VDAC in either of the two closed states did not. Modifications resulting in the loss of voltage-dependence occurred primarily when anhydride was added to the negative side of the membrane irrespective of which closed state the VDAC was in indicating that the accessibility of the gating charges alternated between the *cis* and *trans* sides as the channel's conformation was changed from one closed state to the other. Despite the pronounced asymmetry, in general the resulting channels behaved in the same way in response to either positive or negative fields. A model consistent with the results is presented which proposes that the same gating charges are responsible for channel closure at both positive and negative fields.

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

Introduction

Many proteins able to form water-filled pores in membranes have been described by electrophysiologists. Some of these are under voltage control, i.e. the transmembrane voltage has a profound influence on the probability of finding the channel in the open or closed state. The molecular basis for this voltage control in endocytic[1] channel-formers is still unclear despite much progress in elucidating the mechanisms used by ectocytic[1] channel-formers. The aim of this paper is to elucidate this mechanism for an endocytic channel-former, VDAC.

The outer membranes of all mitochondria tested to date contain proteins which form large channels (Parsons, Williams & Chance, 1966; Mannella & Bonner, 1975; Colombini, 1979; 1980*b*; Zalman, Nikaido & Kagawa, 1980; Freitag, Neupert & Benz, 1982; Linden, Gellerfors & Nelson, 1982; Roos, Benz & Brdiczka, 1982). These channel-formers were called VDAC (Schein, Colombini & Finkelstein, 1976) because of their properties of voltage-dependence and preference for anions. VDAC channels are in their highest conducting state at low transmembrane voltages (<10 mV) but occupy low conducting states at high voltages (>40 mV). In the accompanying paper we have shown that succinic anhydride modifies the channels causing loss of voltage dependence and changing the channel's preference from anions to cations. In this paper, we will use the anhydride reaction to explore the nature of the voltage dependence and attempt to locate the gating charges. A preliminary report of this work has been published (Doring & Colombini, 1984).

Materials and Methods

Planar phospholipid membranes, consisting of soybean phospholipids, were generated following the 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 dissolved in 0.7% Triton X-100, 20% DMSO, 0.5 M KCl, 0.05% Na^+ azide, 1 mM HEPES (Na^+ salt), pH 7.0. The membrane was formed in 1.0 M LiCl, 5 mM $CaCl_2$, 50 mM MOPS (Li^+ salt), pH 7.2. An aliquot of VDAC-containing solution (usually 5 to 10 μl) was added to the aqueous phase bathing the *cis* side of the membrane. If desired, channel insertion could be slowed down or arrested by adding 50 μl of 10 mg/ml Concanavalin A to the *cis* side. (Con A binds to VDAC and inhibits its insertion into the membrane; Colombini, 1980*a*.)

[1] Endocytic refers to channel-formers produced by a cell and inserted into its own membranes. These channel-formers are not normally released into the environment. Ectocytic channel-formers arrive to the cell surface from the environment. They may be man-made or may have been released into the environment by other cells. Ectocytic channel-formers include certain antibiotics, toxins and even chemical messages which form channels once inserted into the membrane of the target cell. The same terms could be used to refer to carriers.

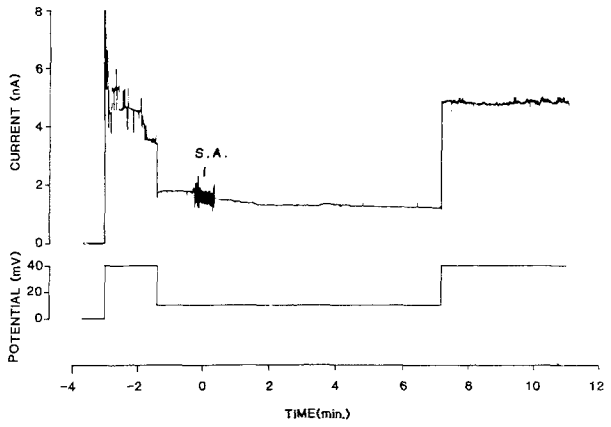


Fig. 1. Low field experiment; succinic anhydride added to the *cis* side. The membrane was made under the conditions described in Materials and Methods. The lower tracing shows the electrical potential at which the membrane was clamped (*cis* side minus *trans* side) and the upper trace is a record of the current flow. The time scale was set at zero at the point at which succinic anhydride was added (S.A.)

Prior to anhydride addition, VDAC was placed into the open or closed state by applying the appropriate electric field. (The sign of the field was defined as the sign of the potential on the *cis* side of the membrane.) A low field (10 mV transmembrane) was used to modify VDAC in the open state while a high field (±40 or 50 mV transmembrane) was used to hold the channels in a closed state. Twenty microliters of succinic anhydride (167 mg/ml dimethylsulfoxide) were added to the side of the membrane indicated in the Figures or text. After waiting for several minutes for most of the anhydride to be hydrolyzed (the anhydride has a half-life of about 2 min in an aqueous phase) the properties of the modified channels were examined. Although anhydride hydrolysis caused acid to be formed, sufficient buffer was present so that the pH never fell below 6.7.

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

Results

VDAC channels were inserted into planar phospholipid membranes as described in Materials and Methods. The channels were added to the aqueous phase bathing one side of the membrane. This side was defined as the *cis* side. Modification with succinic anhydride was performed under either high or low electric field. The voltage on the *cis* side was used to designate the sign of the field. High electric field experiments were performed with either positive or negative fields and with anhydride added either to the *trans* or *cis* side.

A) Low Field Experiments

As reported in the accompanying paper, the addition of succinic anhydride to VDAC channels in the open state resulted in a loss of voltage-dependence and a reversal of ion selectivity. Figure 1 shows the consequences of anhydride addition to the *cis* side in the presence of a low positive field. The membrane contained seven channels which closed in a normal manner in the presence of a high positive field. (Closed VDAC channels are conductive and therefore account for the current level observed after channel closure.) The field was reduced prior to anhydride addition to allow the channels to open. (Because of VDAC's fast kinetics of opening (Colombini, 1979) the opening relaxation curve was not detected due to low time resolution.) Following the addition of succinic anhydride, no channel closure was observed. A reduction in conductance was observed, however. It occurred in small increments indicating that the conductance of each channel was reduced, on the average, by 34%. When the field was increased once again, the modified channels did not close. In longer records and at higher fields, a small amount of closure was observed. The same behavior was observed with positive or negative fields. Similar results were obtained with anhydride added to the *trans* side.

B) High Positive Field, *Cis* Anhydride Addition

The experiments were performed as in A above except that anhydride was added to the *cis* side while VDAC channels were kept closed by means of a high positive field. In Fig. 2*a* the membrane contained a single channel. Prior to anhydride addition the channel closed rapidly in response to a positive field. The channel remained closed throughout the anhydride treatment period but reopened when the field was reduced. Panel *b* shows similar results with a multi-channel membrane. In this case some channels may have opened during the treatment period. However, the results agree very well with those obtained with single channels. The voltage dependence of the channels was reduced and their open-state conductance was reduced by 40 to 50%.

C) High Positive Field, *Trans* Anhydride Addition

In these experiments, the anhydride was added to the *trans* side while the channels were kept closed by means of a high positive field. Two types of results were obtained, exemplified by Fig. 3*a* and *b*. The channels often opened during the reaction period resulting in channels with essentially no voltage dependence (Fig. 3*a*). In this case the open-channel conductance was only slightly changed (up to 20%). Alternatively, the channels might open only briefly

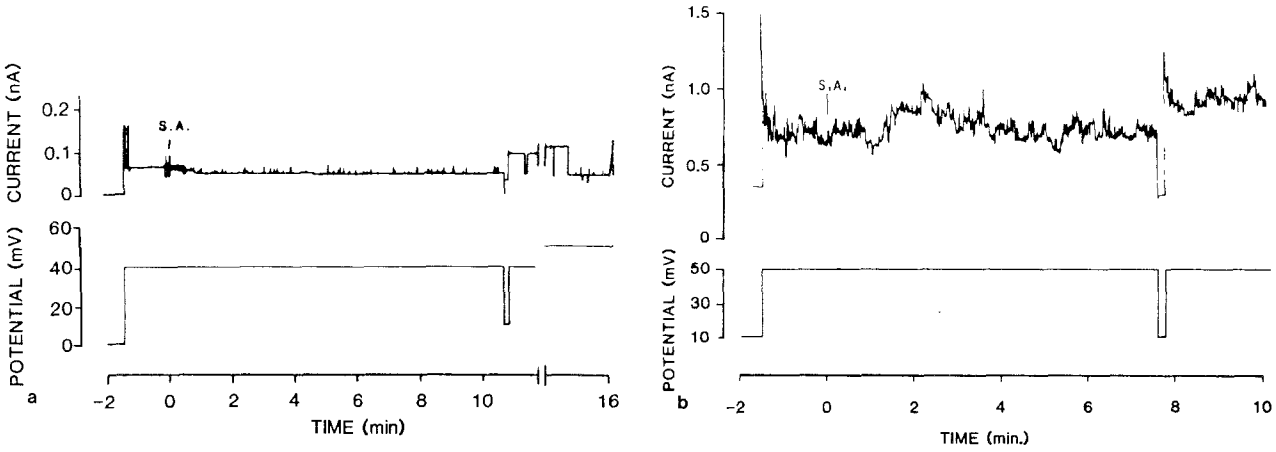
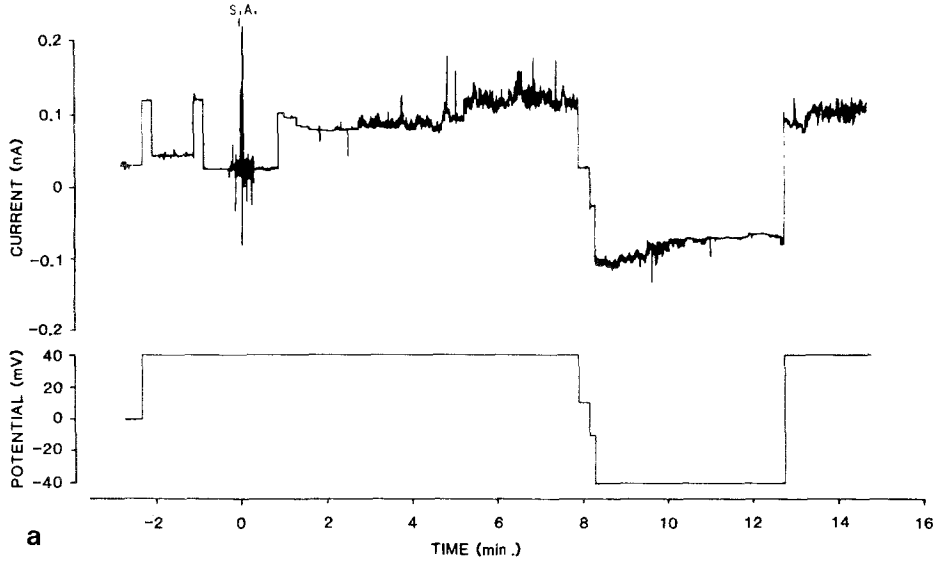


Fig. 2. High positive field experiments; succinic anhydride added to the *cis* side. A single-channel experiment (*a*) and a twelve-channel experiment (*b*) are illustrated. The layout of the Figure is the same as described in Fig. 1



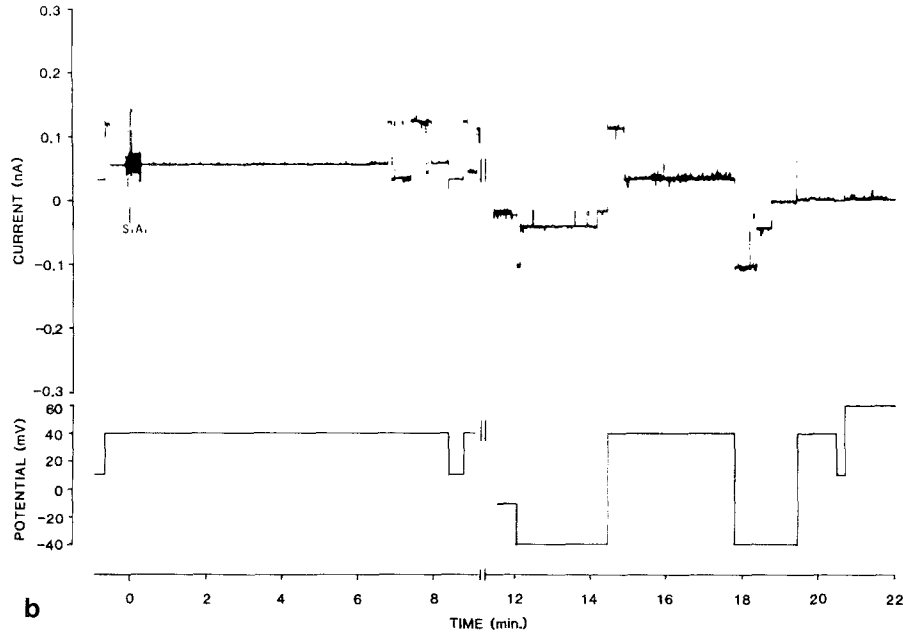


Fig. 3. High positive field experiments; succinic anhydride added to the *trans* side. Two single-channel experiments are illustrated. In *a* the channel loses its voltage-dependence while in *b* it does not. The layout of the Figure is the same as described in Fig. 1

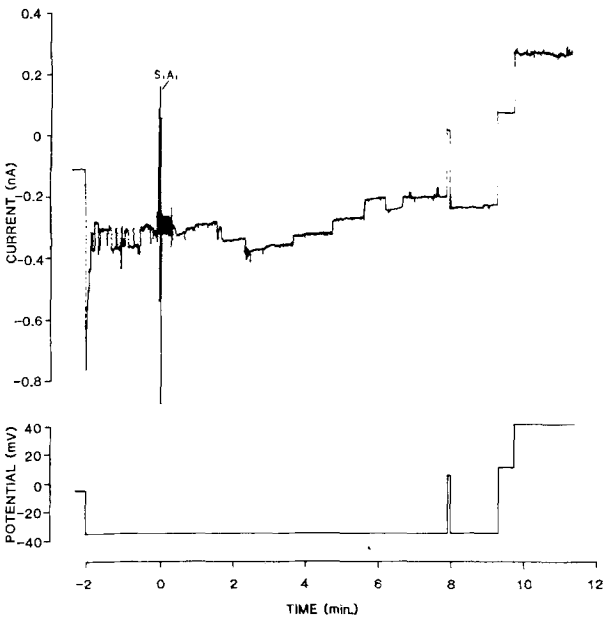


Fig. 4. High negative field experiment; succinic anhydride added to the *trans* side. The layout of the Figure is as described in Fig. 1

(Fig. 3*b*) resulting in channels with virtually unchanged voltage dependence. The probability of obtaining one of these two results was approximately 50%. At times, in response to an applied field, the modified channels would close to a virtually zero conductance state. This transition was usually, but not always, irreversible (Fig. 3*b*).

D) High Negative Field, *Trans* Anhydride Addition

In these experiments the anhydride was added to the *trans* side while the channels were kept closed by means of a high negative field (Fig. 4). This treatment usually resulted in the channels remaining closed even after the field was reduced at the end of the treatment period. The conductance of the modified channels was reduced by about 70%. A number of transitions often occurred during the reaction period whose significance is unclear.

E) High Negative Field, *Cis* Anhydride Addition

In these experiments the anhydride was added to the *cis* side while the channels were kept closed by means of a negative field. The results of this treatment were rather variable. In Fig. 5*a* a single channel was modified. At the end of the modification period, the field was reduced but the channel appeared to remain closed (analogous to positive field, *trans* anhydride addition). The conductance was reduced by 67%. At very high transmembrane voltages (90 mV) it could be completely closed but when this occurred the channel would not easily reopen (analogous to Fig. 3*b*). In panel *b* a different result was obtained. Of the two channels in the membrane, one opened during the reaction period while the other opened when the field was reduced. The overall conductance was reduced by 36%. Both channels would not reclose except at very high voltages (70 mV). Finally in panel *c*, after modification at least some of the channels retained some voltage dependence. The low-field conductance was reduced by 38%. The membrane in panel *c* differed from those in *a* and *b* in that it contained 18 channels. We have found that membranes containing many channels are much more refractory to succinic anhydride modification (of their voltage-dependence) by this procedure. Membranes containing many more channels were even more refractory.

Table 1. Voltage dependence of modified VDAC

Modification conditions: Applied field:	Positive		Negative	
Anhydride addition:	*cis*	*trans*	*cis*[a]	*trans*
Voltage dependence:	Percent of total observations:			
Unchanged		50		
Small change	75			9
Lost		50	70	
Channels remained closed			30	91
Number of observations	18	12	23	11

[a] Only experiments with membranes containing one or a few channels are summarized here.

Table 1 summarizes how anhydride addition, under high field conditions, affected VDAC's voltage-dependence. The degree of modification depended strongly on the reaction conditions. Loss of voltage dependence was most pronounced when anhydride was added to the *trans* side under positive field conditions or to the *cis* side under negative field conditions. Modification under negative field conditions often resulted in channels that remained in what appeared to be a closed state. These channels were rather unresponsive to changes in transmembrane field. Although this unresponsiveness could be interpreted as loss of voltage-dependence, we prefer to interpret the result as a kinetic block to the interconversion between the open and closed conformation (*see* Discussion). The very low conductance of these modified channels (65 to 70% reduction in conductance as compared to the unmodi-

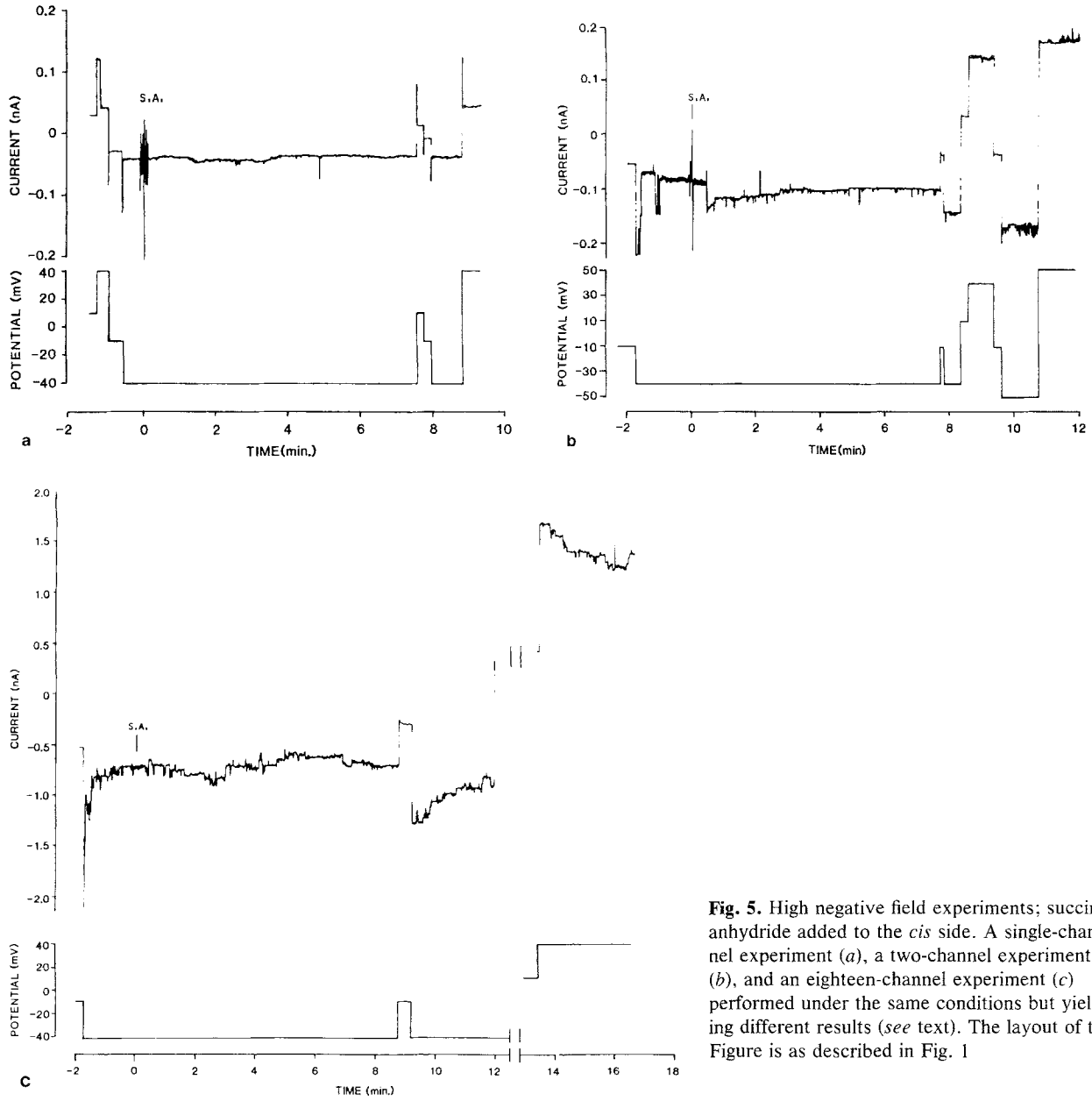


Fig. 5. High negative field experiments; succinic anhydride added to the *cis* side. A single-channel experiment (*a*), a two-channel experiment (*b*), and an eighteen-channel experiment (*c*) performed under the same conditions but yielding different results (*see* text). The layout of the Figure is as described in Fig. 1

fied open state, Table 2) is consistent with this interpretation. However, a more compelling argument can be made by examining single-channel records such as Fig. 5*a*. There is no indication of the single-channel opening during the modification period or after the field was reduced. (The transients seen in Fig. 5*a* are capacitive transients.)

VDAC conductance after anhydride modification fell into rather well-defined groups (Table 2). There appears to be no correlation between a reduction in open-state conductance and loss of voltage dependence. The reduction in open-state conductance was small for *trans* addition of anhydride in the presence of a positive field. Under these condi-

Table 2. Conductance change of modified VDAC

Modification conditions:				
Applied field:	Positive		Negative	
Anhydride addition:	*cis*	*trans*	*cis*[a]	*trans*
Conductance decrease:	Percent of total observations:			
0–20%	25	100		9
35–40%			70	
40–50%	75			
65–70%			30	91
Number of observations	18	12	23	11

[a] Only experiments with membranes containing one or a few channels are summarized here.

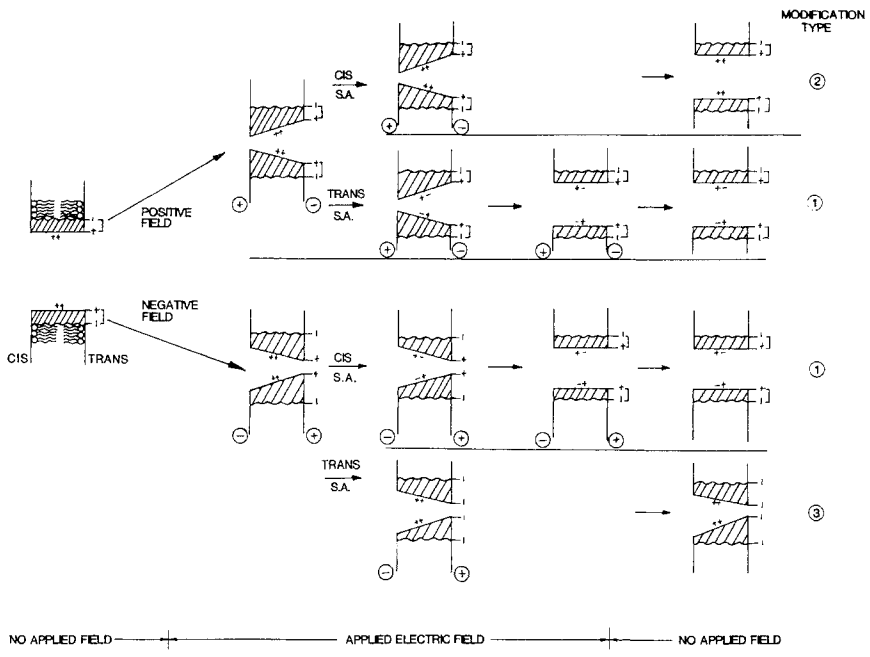


Fig. 6. A voltage-gating mechanism proposed to explain the results obtained. The hatched areas are meant to represent cross-sections (perpendicular to the plane of the membrane) of channels spanning a membrane. The left side of the Figure illustrates channels in the open state and in closed states induced by positive and negative fields. The positive charges within the channel are the gating charges modified by succinic anhydride. The charges on the *trans* end of the channel are postulated to form a salt bridge in the open state and in the closed state in the presence of a positive field but not in the presence of a negative field. The succinic-anhydride-modified forms are shown on the right side of the Figure. Four reaction sequences are shown resulting from anhydride addition to the *cis* (first and third sequence) or the *trans* (second and fourth sequence) side of the membrane

tions, only half the channels tested lost their voltage dependence. The conductance decrease was perhaps only slightly greater in those channels whose voltage dependence was lost. Similarly, when *cis* anhydride addition reduced channel conductance by about 40%, voltage-dependence loss in these channels was dependent on the sign of the applied field.

When VDAC is inserted into planar phospholipid membranes under the conditions described in Materials and Methods, each channel will close under both positive and negative fields. The probability of a channel being closed is given by the magnitude of the field and, to a first approximation, is independent of the sign of the field (with membranes made as described in Materials and Methods). The second sentence of this paragraph also holds for most of the modified channels we observed irrespective of how the channel was modified. In Fig. 5*b*, for example, anhydride was added to the *cis* side in the presence of a positive field but the modified channels failed to close under both positive and negative fields.

Discussion

Modifications induced by succinic anhydride seemed to fall into four categories: 1) modification of the groups which normally respond to the transmembrane field causing the channel to open or close (i.e. modification of the gating charges); 2) modifications resulting in negligible effects on channel conductance or voltage dependence; 3) modifications which result in steric constraints which reduce or inhibit conformational changes; 4) modifications which primarily effect the single-channel conductance. Type 2 modifications can be ignored. Type 1 should result in channels with reduced voltage dependence. These were observed with *trans* anhydride addition under positive fields and *cis* addition under negative fields. Modifications in the presence of negative fields, which resulted in channels which remained closed, were probably type 3 modifications. Type 4 modifications occurred most often when anhydride was added to the *cis* side.

From the types of modifications induced in VDAC under the different conditions it is possible to make some conclusions on the location of the gating charges in the different conformational states. The largest numbers of type 1 modifications (gating charge modification) occurred when anhydride was added to the *trans* side under positive field conditions or to the *cis* side under negative field conditions. In these cases the voltage-dependence was almost completely lost. If the *cis* side of the membrane was made positive (positive field) then positive charges should be driven to the *trans* side where they could react with anhydride. Conversely, a negative field should drive positive charges to the *cis* side. The observations are therefore consistent with these expectations.

The conformation achieved by VDAC in the presence of negative fields is almost certainly differ-

ent from that achieved under positive fields. The simplest explanation might invoke two gating systems with two different sets of gating charges. Indeed, the Na^+ channel in the squid axon which closes at both positive and negative fields exhibits clearly different voltage-dependent properties for each of the two types of closure. Therefore we were surprised to find that in most cases when we tested the modified channels with positive and negative fields the results were essentially the same. The channels seemed to lose voltage dependence in both gating systems to the same extent as if only one set of gating charges were present.

A schematic model consistent with the observations is shown in Fig. 6. The channel is a cylinder in the open state and becomes cone-shaped in either closed state. The two closed states are different in that different ends of the channel-former become constricted in the two states. The same gating charges are used for both voltage-dependent conformational changes. In the presence of a field the charges stabilize one of the cone-shaped conformations via two voltage-dependent energy terms: 1) the alignment with the field of the dipoles perpendicular with the walls of the channel results in a reduction in the energy level of the closed state; 2) a change of the potential at the charges, due to a constriction of the pore at one end, results in a change in the energy level of the charges as if the charges had moved through a voltage difference (Colombini, 1984). The location of these charges on the walls of the channel also account for VDAC's preference for anions over cations. This dual function for these charges is supported by the finding (*see* accompanying paper) that succinic anhydride alters voltage dependence and channel selectivity simultaneously.

Using this simple model it is possible to explain some of the results reported in Table 1. The gating charges in the channel are preferentially modified by anhydride addition to the *trans* side in the case of positive field and to the *cis* side for negative field. In an effort to explain type 3 modifications, we have postulated that ion pairs exist on the *trans* side which as such are relatively refractory to reaction with anhydride. Under positive fields their structure remains relatively undisturbed because it is the *cis* side that pinches off. However, under negative fields the *trans* side constricts forcing the ion pair to pull apart (or to be exposed to the aqueous environment) resulting in an increase in anhydride reactivity. Once the anhydride has converted the positive group to a negative group, a return to the open configuration is not possible so the channel remains closed. This aspect of the mechanism is rather arbitrary and other structural changes could be postulated to account for the type 3 modifications.

The data summarized in Table 1 is not as clear cut as indicated by the mechanism portrayed in Fig. 6. Anhydride added to the *cis* side under positive field conditions does result in some modification of VDAC's voltage-dependence. Since VDAC's closed state is not totally closed, anhydride could pass through the narrow portion and react with the gating charges. Indeed VDAC's closed state will allow succinate to pass but at a tenfold lower rate (M.C., *unpublished results*). Nevertheless, the results show that type 1 modifications occurred preferentially when anhydride was added to one side of the membrane. Hence the proposed mechanism is consistent with the results.

We have not tried to explain the conductance changes shown in Table 2, except for the large changes observed under negative field conditions. As indicated above, the large changes probably represent channels which have remained in the closed conformation. Since these type 4 modifications do not appear to correlate with voltage-dependence changes, it is difficult to attribute any particular function to the structures that were modified.

In summary, the results indicate that one set of gating charges are responsible for providing the energy needed to close VDAC at both positive and negative fields. The gating charges are accessible preferentially from one side of the membrane when VDAC is in one of the closed conformations. The preferential accessibility of the gating charges alternates between the *cis* and *trans* sides as the conformation changes from one closed state to the other. The direction of this change in accessibility is consistent with positive gating charges. Whether this change in accessibility occurs prior to channel closure or occurs concomitantly with closure we cannot say. The proposed gating mechanism predicts that both events should occur simultaneously. Hence the gating charge associated with channel closure should occur at the same time as the change in ionic current. Although the proposed mechanism makes a number of predictions, one interesting prediction is that if all the gating charges were converted to negative charges by anhydride modification, the voltage dependence should be restored.

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