J. Membrane Biol. 99, 187–196 (1987)

The Journal of
Membrane Biology
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Voltage Gating in VDAC is Markedly Inhibited by Micromolar Quantities of Aluminum

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Summary. The mitochondrial outer membrane contains voltage-gated channels called VDAC that are responsible for the flux of metabolic substrates and metal ions across this membrane. The addition of micromolar quantities of aluminum chloride to phospholipid membranes containing VDAC channels greatly inhibits the voltage dependence of the channels' permeability. The channels remain in their high conducting (open) state even at high membrane potentials. An analysis of the change in the voltage-dependence parameters revealed that the steepness of the voltage dependence decreased while the voltage needed to close half the channels increased. The energy difference between the open and closed states in the absence of an applied potential did not change. Therefore, the results are consistent with aluminum neutralizing the voltage sensor of the channel. pH shift experiments showed that positively charged aluminum species in solution were not involved. The active form was identified as being either (or both) the aluminum hydroxide or the tetrahydroxoaluminate form. Both of these could reasonably be expected to neutralize a positively charged voltage sensor. Aluminum had no detectable effect on either single-channel conductance or selectivity, indicating that the sensor is probably not located in the channel proper and is distinct from the selectivity filter.

Key Words membrane · aluminum · channel · mitochondrion · voltage dependence · VDAC

Introduction

Aluminum is present in small amounts in most mammalian tissues, but it has no known biological function. Far from being innocuous, it has been shown to have a variety of toxic effects. These toxic effects occur in many organs [e.g., kidney (Burnatowska-Hledin et al., 1985*a*), brain (Perl, 1985) and parathyroid (Mayor & Burnatowska-Hledin, 1983)] and disrupt a broad spectrum of cellular processes including membrane transport systems [Ca^{2+} (Siegel & Haug, 1983; Burnatowska-Hledin & Mayor, 1984), phosphate (Dousa & Kempson, 1982; Burnatowska-Hledin et al., 1985*a*), choline uptake and Na-K-ATPase activity (Lai et al., 1980)] and enzymatic activity [bone phosphatases (Lieberherr et al., 1982) and brain cytosolic and mitochondrial hexokinases) (Solheim & Fromm, 1980; Lai & Blass, 1984; Lai et al., 1985)].

Aluminum toxicity has been implicated as a possible causative agent in a variety of physiological disorders: minor skin lesions (Fisher, 1984), Alzheimer's disease (Shore & Wyatt, 1983; Perl, 1985), amyotrophic lateral sclerosis and parkinsonism-dementia (Klatzo, Wisniewski & Streicher, 1965; Terry & Pena, 1965; Perl & Brody 1980*a,b*; Perl et al., 1982; Garruto et al., 1984). The osteomalacia, anemia and dementia often associated with long-term hemodialysis have also been linked to aluminum toxicity (Parkinson, Ward & Kerr, 1981; Verbueken, et al., 1984).

Although there is no obvious common mechanism responsible for its wide-spread toxicity, many reported toxic effects of aluminum involve alterations in cellular energy levels and mitochondrial function. Aluminum depresses brain glycolysis through inhibition of both cytosolic and mitochondrial hexokinase activity as well as lactate production (Solheim & Fromm, 1980; Lai & Blass, 1984; Lai et al., 1985). The inhibition of the cytosolic enzyme is due to formation of an aluminum-ATP complex which competes for the enzyme's active site, but direct aluminum binding has also been suggested (Solheim and Fromm, 1980; Viola, Morrison & Cleland, 1980; Neet, Furman & Hueston, 1982). Finally, aluminum depresses mitochondrial respiration (state 3) and increases mitochondrial permeability (Burnatowska-Hledin et al., 1985*b*).

It is widely held that mitochondrial substrates and their major product, ATP, cross the outer mitochondrial membrane via water-filled channels called, VDAC. VDAC is a channel-forming protein located in the outer mitochondrial membrane of all eukaryotic organisms studied (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; Mannella & Colombini, 1984; Nakashima et al., 1986; Smack & Colombini, 1985). Its major characteristic properties are: (i) the aqueous pathway formed by the channel is large [1.5 to 2 nm in radius (Colombini, 1980*b*; Mannella, 1982)] and highly conductive (4.2 nS in 1 M KCl); (ii) it is voltage dependent, existing preferentially in a high conducting state (open) at low voltages and in low conducting states (closed) at high applied voltages (Schein et al., 1976; Colombini, 1979, 1980*a,b*); (iii) the channel is weakly anion selective (Colombini, 1980*b*).

The mechanisms by which VDAC senses and responds to an electric field (voltage dependence) and selects among ions is not understood. Recent findings have lead to the formulation of a working model (Bowen, Tam & Colombini, 1985; Doring & Colombini, 1985*a,b*; Colombini, 1986). The model proposes that the protein, in the open state, forms a cylindrical water-filled pore. This is converted into a cone-shaped structure when the channel enters a closed state. In this model, both voltage dependence and ion selectivity result from the presence of a single set of positive charges lining the walls of the aqueous pore.

In this paper we present evidence that low levels of aluminum inhibit the voltage-dependent closure of the VDAC channels in a dose-dependent manner. Probing the nature of this inhibition has provided new information about the structure and function of the VDAC channel.

Materials and Methods

VDAC Preparation

VDAC was obtained from the mitochondrial membranes of a wall-less (slime) mutant of *Neurospora crassa* (ATCC #32360). Cells were maintained, cultured and harvested as described by Mannella (1982). Mitochondrial membranes were isolated by previously described methods with slight modification (Mannella, 1982). Briefly, mitochondria were isolated by differential centrifugation and hyposmotically lysed to remove soluble proteins. Membranes were concentrated by centrifugation and resuspended in 1 mM KCl, 1 mM HEPES (pH 7.0), and 15% (vol/vol) dimethylsulfoxide (DMSO) to a final protein concentration of approximately 3 mg/ml. The bulk of the membrane preparation was stored at −70°C, and aliquots were transferred to −20°C for short-term storage prior to use.

General Methods

The following methods were generally employed throughout the study. Modifications and details relevant to specific portions of the work appear in Results with explanations. Experiments were conducted on planar phospholipid membranes formed by the monolayer method of Montal and Mueller (1972) as described by Schein et al. (1976). Membranes were generated from soybean phospholipids (Type II-S, Sigma Chemical Co.) purified and stored as previously described (Kagawa & Racker, 1971). A known volume of aqueous solution (usually 5 ml) containing 1.0 M LiCl (or KCl) and 5.0 mM $CaCl_2$ bathed both sides of the membrane. All experiments were conducted under voltage-clamp conditions using a pair of calomel electrodes to interface with the aqueous phases. The *trans* side of the membrane was maintained at virtual ground so that all potentials refer to the *cis* side.

The VDAC-containing membrane solution was treated with Triton X-100 (1% final conc.) at room temperature for at least 20 min prior to use. A small aliquot of this solution (5–10 μl) was added to the *cis* aqueous phase. Stirring began just prior to the addition of VDAC and was continued for at least 30 sec. With a low applied voltage (−10 mV) the spontaneous insertions of VDAC channels were monitored as step-wise increases in current. Current flow was recorded on a Kipp & Zonen BD41 chart recorder. The presence of typical VDAC behavior was verified before any further additions were made to the system. A closure-inducing voltage (± 40 or 50 mV) was applied and the rate and extent of the membrane current decrease was observed. All subsequent additions ($AlCl_3$, buffers, etc.) were made to both the *cis* and *trans* sides with stirring during and, for at least 30 sec., following the additions. A −10 mV potential was applied during additions in order to monitor the membrane conductance.

The aqueous phase was buffered with 10 mM Tris-HCl, pH 7.0, by the addition of 50 μl of a 1.0 M stock solution. At this concentration the aqueous phase pH remained above 6.85 even with the highest $AlCl_3$ concentration used, 100 μM. A 10 mM $AlCl_3$ stock solution was prepared from reagent grade anhydrous $AlCl_3$ (Aldrich Chemical Co.). Other chemicals were reagent grade and all solutions were prepared in double glass-distilled water.

Data Analysis

The time constant of channel closure, τ, was quantified from current records of multi-channel membranes as the time required for membrane current to decrease to $1/e$ of the total current change following a voltage step. The extent of channel closure was quantified as the percent the current decreased from the instantaneous open channel current. Calculations were based on the assumption of steady-state current after 2 min at the applied voltage.

The analysis of the effect of $AlCl_3$ additions on the voltage-dependent properties of VDAC was performed using a modification of the methods introduced by Ehrenstein, Lecar and Nossal (1970) for the analysis of EIM channels and used by Schein et al. (1976) for VDAC channels. This analysis assumes that the channels can only be in either the "open" or "closed" state and that they are at equilibrium at each voltage. Equation (1) is based on the Boltzmann distribution and describes the probability of VDAC channels being in the open or closed state at a given voltage applied across a multi-channel membrane:

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

In this equation G is the membrane conductance at any voltage, V, G_{max} is the maximum conductance (when essentially all the

channels are open) and G_{min} is the minimum conductance (when essentially all the channels are closed). R, T and F are the gas constant, temperature in degrees Kelvin and Faraday's constant, respectively. V_o is the voltage at which half the channels are closed and n is a measure of the steepness of the voltage-dependent closure. If the applied voltage, V, is equal to V_o then there is no energy difference between the open and closed states and the probability of the channels being open or closed is equal. At voltages less than V_o the energy of the open state is less than that of the closed state and, therefore, the probability of the channels being open is greater. Conversely, with applied voltages greater than V_o the closed state of the channels is more probable. In the absence of an applied field, $V = 0$, the energy difference between the two states is the intrinsic conformational energy difference and the energy needed to compensate for this energy difference is given by nFV_o.

For this analysis, the measurements of multi-channel membrane current recorded as a function of voltage were converted to conductance measurements as a function of voltage using a Hewlett-Packard digitizer and HP-85 computer. The n and V_o values were then obtained from the slope and y-axis intercept of the plot of $\ln (G - G_{min}/G_{max} - G)$ *vs.* voltage, respectively.

Obtaining correct estimates of n and V_o, therefore, depends on having accurate G_{max} and G_{min} values. In these experiments it was difficult to obtain G_{min} values directly in all situations because with the addition of $AlCl_3$ a larger voltage must be applied to achieve G_{min} (*see* Results). These high voltages were prohibitive because they cause membrane breakdown. However, experiments with only a few channels in the membrane indicated that with 100 μM added $AlCl_3$, the mean open and closed channel conductances were not substantially different from those observed in $AlCl_3$-free controls (open control, 2.8 nS, plus aluminum, 2.8 nS: closed control, 1 nS, plus aluminum, 0.8 nS)[1]. Therefore, it was possible to determine G_{min} for $AlCl_3$-treated channels indirectly from the value of G_{max} after $AlCl_3$ addition. The percent of closure before $AlCl_3$ addition was calculated and used to determine the G_{min} value for the $AlCl_3$-affected membranes from the measured G_{max} value after $AlCl_3$ addition.

Results

Voltage-Dependence Parameters

Voltage-dependent closure is a major property of the VDAC channel. $AlCl_3$-induced changes in this voltage dependence of the channels was studied on multi-channel membranes bathed in buffered (pH 7.0) aqueous phases (*see* Materials and Methods). The rate of channel closure and the extent of closure at fixed voltages which normally induce closure was determined. The voltage was stepped from an open channel voltage, 0 mV, to a voltage that induces rapid closure in unmodified channels, 50 mV. The voltage was maintained at 50 mV until no further decrease in current was evident (at least 2 min). The applied voltage was then returned to an open channel voltage for approximately 2 min. $AlCl_3$ was then added to a final concentration of 1.0 μM and a closure voltage applied as described above. The process was repeated with sequential $AlCl_3$ additions. The behavior of channels bathed only in buffered salts served as the control and the data normalized relative to it.

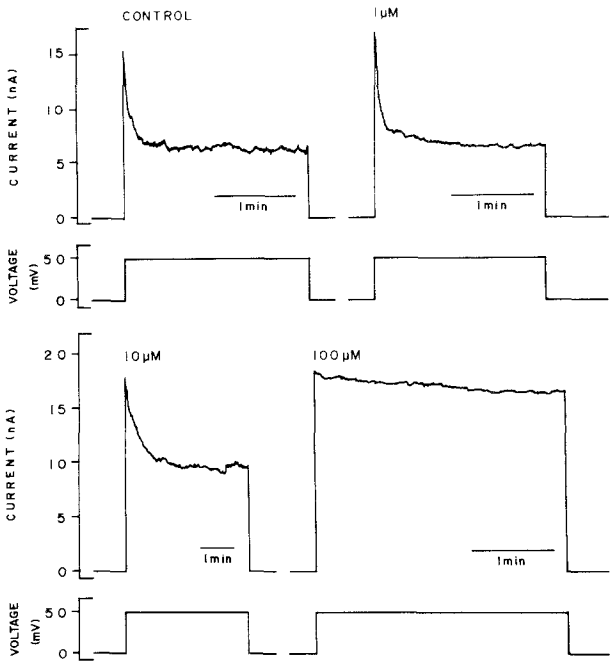

Fig. 1. Low levels of aluminum chloride decrease both the rate of channel closure and the voltage-dependent decrease in membrane conductance at 50 mV. Sequential additions of $AlCl_3$ were made to a single membrane containing many channels bathed in 1 M LiCl, 5 mM $CaCl_2$, 10 mM Tris-HCl at pH 7. Tracings are the change in total membrane current in response to the voltage step indicated. Note the time scale change for the 10-μM aluminum chloride addition

Figure 1 is a typical experiment illustrating the effect of increasing $AlCl_3$ concentrations on the voltage-dependent closure of VDAC. In the absence of $AlCl_3$, the conductance across a membrane containing many VDAC channels decreased about 60% with the application of a high (50 mV) potential (Fig. 1, Control). $AlCl_3$ additions as low as 10 μM (in some experiments, *not shown*, 1-μM levels produced changes in closure rates) reduced this voltage-dependent conductance as well as increased the time required to achieve a steady-state closed chan-

[1] While there may be some decrease in the closed channel conductance in the presence of aluminum, we used the control closed channel conductance levels in these calculations for two reasons. First, it is possible that the observed reduced closed channel conductance in the presence of aluminum may be a function of the higher voltages required to obtain measurable closure events. And, second, the shift is toward greater channel closure and would not, therefore, alter the qualitative effects or interpretation. If anything, the data presented here may be an underestimation of the aluminum effect on VDAC.

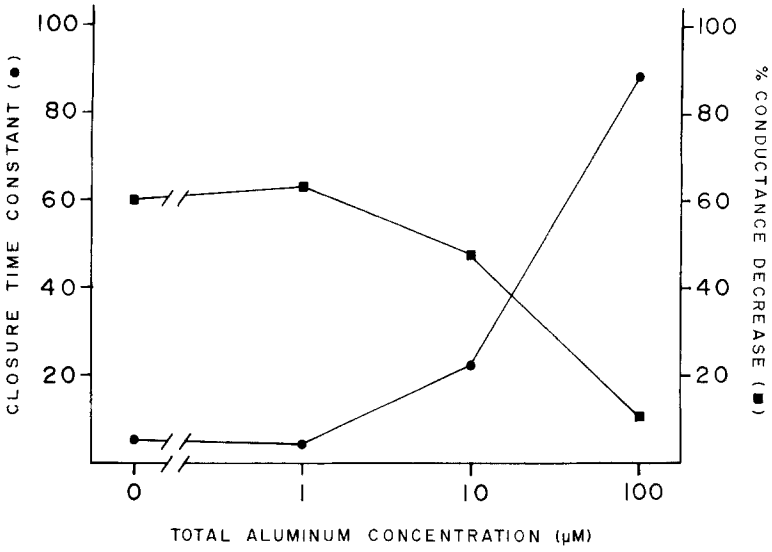

Fig. 2. Aluminum chloride decreases the rate of channel closure (●) and the voltage-dependent conductance decrease at 50 mV (■). Data are from the experiment shown in Fig. 1. The rate of closure was quantified as the time constant, τ. The extent of voltage-dependent closure was quantified as the percent the current decreases from the instantaneous open channel conductance at 50 mV. Calculations were based on the assumption of steady-state current after 2 min

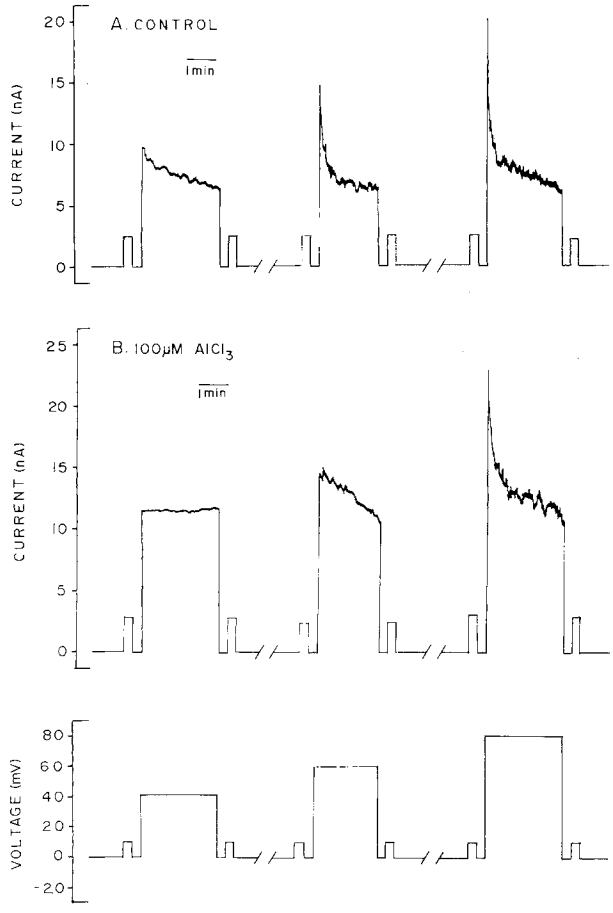

Fig. 3. Aluminum chloride treatment increases the voltage needed to close the channels. Channel closure similar to that observed at 60 mV in the untreated membrane (*A*) is evident at 80 mV in the presence of aluminum (*B*). The experimental conditions were the same as those given in Fig. 1a

nel conductance (Figs. 1, 2). $AlCl_3$ levels of 100 μM virtually eliminated voltage-dependent closure at 50 mV.

Such a net effect could result either from an increase in the closed state conductance of the individual channels or from a reduced probability of the channel being in the closed state. We, therefore, examined the open and closed state conductance properties of single channels in the presence (100 μM) and absence of $AlCl_3$. Open channel conductances were quantified from individual channel insertions observed at ±10 mV. Closed channel conductances were studied in membranes containing a few channels by measuring the step-wise conductance drops resulting from channel closure at high membrane potentials.

The addition of 100 μM aluminum did not alter the mean open channel conductance (control, 2.8 ± 0.3 nS; aluminum, 2.8 ± 0.3 nS). Although channel closure occurred less frequently in the presence of aluminum, it could be observed especially at higher potentials (60–80 mV). The mean closed channel conductance was not increased by aluminum (control, 1.0 ± 0.3 nS; aluminum, 0.8 ± 0.5 nS).

Not only were channels still capable of closing in the presence of aluminum, their probability of closing increased with higher applied potential. This is evident in a multi-channel membrane shown in Fig. 3. Raising the applied voltage above that normally required to close VDAC in the absence of aluminum increased the extent of voltage-dependent closure as well as the rate of closure of aluminum-treated channels (*compare* Fig. 3*A* and *B*). Thus, aluminum does not alter VDAC's closed state conductance, but reduces the probability that the

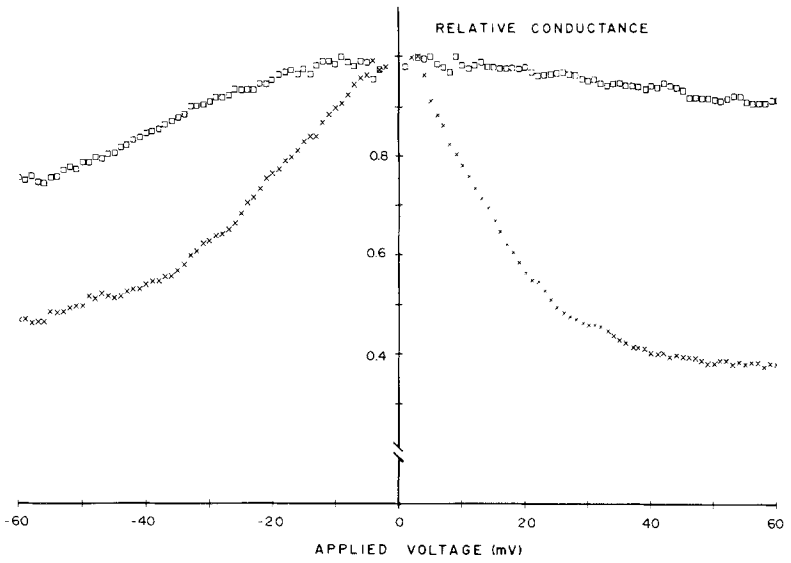

Fig. 4. Aluminum decreases the voltage dependence of VDAC's steady-state conductance. The experimental conditions were as indicated in Fig. 1, except that triangular voltage waves were applied (25 mV/sec.). The current recorded as the voltage decreased was converted to conductance, normalized to the conductance at zero voltage and plotted. Controls, no $AlCl_3$(×) and 100 μM final $AlCl_3$ concentration (□)

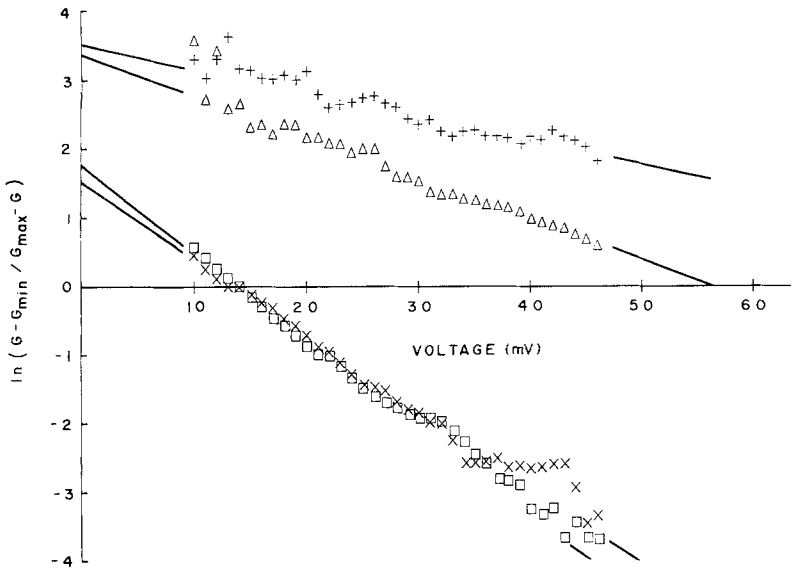

Fig. 5. Linearized membrane-conductance data of a typical experiment. Each conductance point was log transformed as in Eq. (1) and plotted as a function of the applied voltage. A best fit line was drawn through the linear portion of each curve using regression analysis. From Eq. (1), the slope of the line yields the value of n and V_o can be obtained from the y intercept. Control (□): n = 3.2, V_o = 14 mV; 1 μM $AlCl_3$(×): n = 3.0, V_o = 13 mV; 10 μM $AlCl_3$(△): n = 1.5, V_o = 57 mV; 100 μM $AlCl_3$(+): n = 0.9, V_o = 100 mV

channel will be in that closed state at a normal closure voltage.

In order to gain insight into the molecular mechanism by which $AlCl_3$ reduces VDAC's voltage dependence, detailed studies were undertaken to quantitate and characterize the changes in voltage dependence induced by $AlCl_3$. A triangular voltage wave (from 63 to −63 mV at 25 mV per min) was applied to a multi-channel membrane, and the resulting current was monitored. These current values were converted to conductances. Typical results are shown in Fig. 4 for experiments performed in the absence and presence of aluminum. The presence of 100 μM $AlCl_3$ markedly reduced VDAC's voltage-dependent closure over a range of voltages at which extensive channel closure is normally seen (Controls). The reduction in voltage dependence occurs at both negative and positive applied voltages, although it is less pronounced with negative voltages. This asymmetry may have resulted from the fact that we did not try to control the free aluminum concentration and that the VDAC aliquot was added to the *cis* side only.

The membrane conductance versus applied voltage data (such as those in Fig. 4) were fitted to Eq. (1). Samples of log transformed data are shown in Fig. 5. $AlCl_3$ additions decreased the steepness of the voltage dependence, n, and increased the volt-

age required to close half the channels, V_o (Fig. 6). Voltage-dependent closure was minimally affected by the addition of 1 μM $AlCl_3$. However, 10 μM added $AlCl_3$ decreased n by more than 50%, and this decrease in the steepness of the voltage dependence was mirrored by an increase of similar magnitude in the voltage required to open half the channels, V_o. The product of these two parameters, nFV_o, the energy required to close half the channels, was unaffected even at 100 μM (Fig. 6). Since nFV_o was unchanged, the energy difference between the open and closed states was not altered by the addition of $AlCl_3$. Aluminum, therefore, appears to alter specifically the voltage-sensing mechanism of the channels.

Channel Selectivity

A second major feature of the VDAC channel is its preference for anions over cations. Previous work suggested that the same portion of the protein is responsible for both voltage sensing and channel selectivity. Therefore, the possibility that $AlCl_3$ also alters channel selectivity was tested. Ion selectivity was estimated by measuring the reversal potential, which is the voltage required to bring the current across a VDAC-containing membrane to zero in the presence of a salt gradient to drive ion flow. Thus, the degree to which VDAC channels selected chloride ions over potassium ions was determined by comparing the reversal potential in the presence and absence of $AlCl_3$. The current through a multichannel membrane formed across a 10-fold salt gradient was monitored. A potential was then applied to bring the current to zero (Fig. 7). The closure of VDAC channels was observed at a 50-mV applied potential, demonstrating that the channels were behaving normally. $AlCl_3$ (100 μM) was then added, and the reversal potential was determined. Again an attempt was made to close the channels by applying 50 mV.

The reversal potential of unmodified channels was 10 mV and was not changed by the addition of

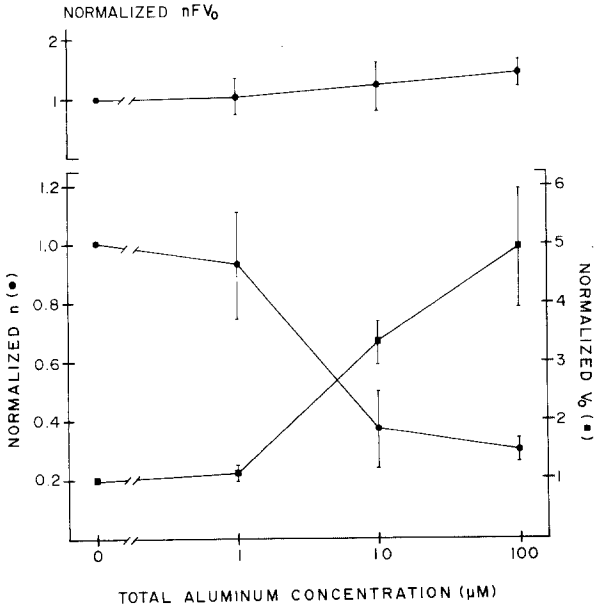

Fig. 6. Aluminum changes the voltage-dependence parameters of VDAC. Plots, such as those shown in Fig. 5, were generated for several experiments and analyzed (*see* Materials and Methods). With increasing aluminum concentration, the steepness of voltage dependence, n (●), decreases; the voltage at which half the channels are open, V_o(■), increases, and the energy difference between the open and closed states in the absence of an applied field, nFV_o, remains essentially unchanged. Mean of 3, ± SE

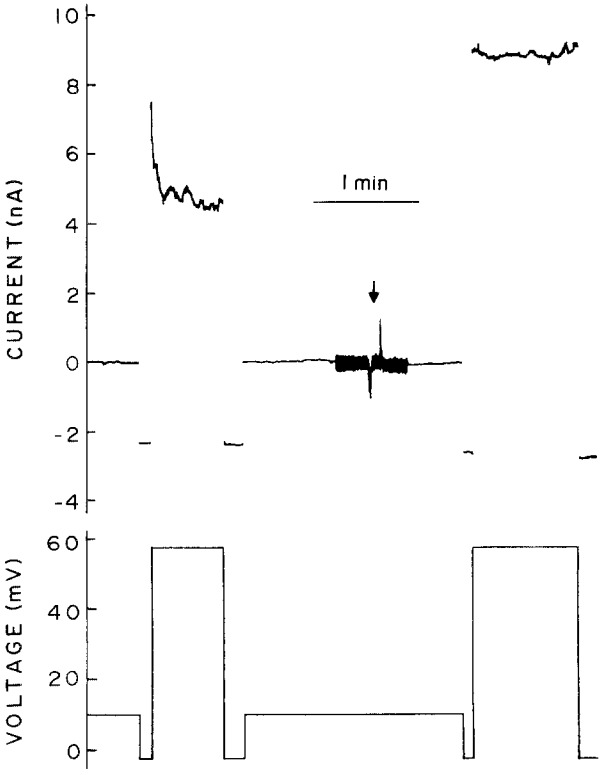

Fig. 7. Aluminum does not affect the degree to which VDAC can select for chloride over potassium. A multi-channel membrane was formed in the presence of a 10-fold KCl gradient (1 M KCl *vs.* 0.1 M KCl; 5 mM $CaCl_2$, 10 mM Tris-HCl at pH 7 also present), resulting in current flow in the absence of an applied field. An 11-mV potential was applied to bring the current to 0. The application of 60 mV resulted in normal channel closure. At the arrow, $AlCl_3$ (100 μM final conc.) was added. The current did not change, indicating no change in ion selectivity. A 60-mV voltage applied after the $AlCl_3$ treatment did not result in channel closure

100 μM $AlCl_3$ (Fig. 7). Furthermore, Fig. 7 illustrates that, while the addition of $AlCl_3$ caused the typical loss of voltage-dependent closure, ion selectivity remained unchanged.

Effective Aluminum Specie

The aluminum species present in aqueous solution following $AlCl_3$ addition are a function of pH. Using published stability constants for the most commonly occurring aluminum forms, the dominant aluminum specie present in the chamber at a given pH was calculated (Table). At pH 7 hydroxoaluminate ($Al(OH)_4^-$, aluminate) and aluminum hydroxide ($Al(OH)_3$) are the dominant forms, while at pH 4 the aluminum ion (Al^{3+}) predominates. At intermediate pH values, the hydroxoaluminum ion ($AlOH^{2+}$) is prevalent. Therefore, by altering the pH of the aqueous phase the dominant aluminum species could be altered and its effect on the voltage-dependent closure of VDAC studied. The pH was altered by the addition of HCl or NaOH to the bath, and the current through the VDAC-containing membrane was recorded in the presence of a constant closure-inducing potential. Since asolectin membranes are extremely fragile at low pH values, these experiments were conducted on diphytanoyl phosphatidylcholine (DPPC) membranes. Although VDAC channels insert into DPPC membranes quite well, the rate of voltage-dependent closure is typically reduced from that seen with the same protein reconstituted into asolectin membranes.

$AlCl_3$-induced reductions in voltage-dependent closure were routinely observed at pH values between 5 and 7. Figure 8 shows a typical experiment on a multi-channel membrane in which the pH was varied and VDAC's voltage dependence was monitored. The voltage-dependent closure present at pH 7 was nearly abolished in the presence of 100 μM $AlCl_3$. Closure was regained when the aqueous phase was acidified to pH 4. Upon subsequent neutralization, voltage-dependent closure was eliminated once again. The effect, therefore, is aluminum-species specific and is reversible by altering the form of aluminum through pH manipulation.

Table. Aluminum species in solution[a]

pH	Al^{3+}	$Al(OH)^{2+}$	$Al(OH)_2^+$	$Al(OH)_3$	$Al(OH)_4^-$
	(% of total aluminum)				
4	86.7	8.9	4.3	<0.1	<0.1
5	12.5	12.8	62.6	12.5	<0.1
6	<0.1	0.7	32.8	65.5	0.6
7	<0.1	<0.1	4.4	87.0	8.7
8	<0.1	<0.1	0.2	49.9	49.9
9	<0.1	<0.1	<0.1	9.1	91.8

[a] Calculated from dissociation constants (20°C, 0 ionic strength) obtained from Smith & Martell, 1976.

Discussion

This is the first report of a direct aluminum-induced alteration of the action of a specific membrane channel. Although many aspects of the VDAC protein have been studied, the actual mechanism of voltage dependence and ion selectivity remain the subject of intense investigation. Evidence from a variety of steady-state studies have been used to

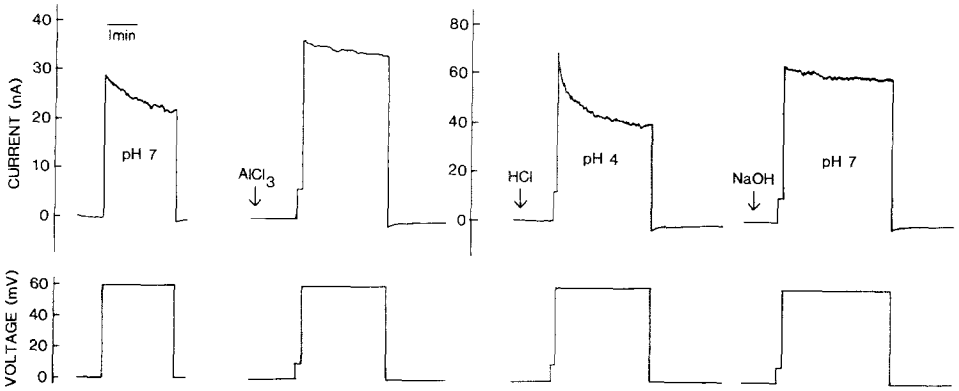

Fig. 8. The pH dependence of the aluminum-induced alteration of VDAC's voltage dependence. These experiments were performed in 1 M KCl, 5 mM $CaCl_2$, and 10 mM Tris-HCl, initially at pH 7. The membranes were made using diphytanoyl phosphatidylcholine. The records were all obtained on a single membrane and proceed chronologically from left to right. The breaks in the record indicate that only the relevant portions of the trace are displayed. Some channel insertion occurred during the experiment, necessitating the indicated scale change. Sufficient HCl and NaOH were added where noted to achieve the indicated pH

formulate a model (Doring & Colombini, 1985*b*; Colombini, 1986). In this model the channel is a cylinder in the open state and assumes a cone conformation in the closed state. It proposes that a single group of positive charges within the channel both responds to the electric field, resulting in voltage-dependent changes in the protein conformation, and serves as the selectivity filter, resulting in preferential anion flow. The model has been consistent with subsequent work. Increasing the pH of the bathing solution reduced the steepness of voltage dependence, again indicating the presence of positive gating charges (Bowen et al., 1985). Furthermore, the p*Ka* values of the titratable groups suggests that the charges are lysine epsilon amino groups. Most recently, it has been possible to use succinic anhydride to convert the proposed positive charges to negative charges (Adelsberger-Mangan & Colombini, 1987). In the course of the titration, the voltage dependence and selectivity were initially lost as the charges were neutralized. As the charges became negative, both phenomena returned. However, following the titration, the channels were cation selective.

Low levels of aluminum interfere with the voltage-gating process in the mitochondrial channel, VDAC. This is important from various perspectives. First of all, it represents the first report of aluminum interfering with the action of membrane channels and one of the few reports dealing with aluminum action at the molecular level. Secondly, if VDAC's voltage-gating turns out to be important in the regulation of mitochondrial function, then low levels of aluminum interfere with this regulation. Thirdly, and of immediate importance, aluminum may serve as a molecular probe of VDAC's voltage-gating mechanism.

Aluminum could inhibit VDAC's voltage-dependent closure in a variety of ways: Firstly, aluminum may bind to the protein in such a manner as to simply prevent channel closure altogether. The observed changes in membrane conductance as a function of $AlCl_3$ addition would simply reflect the normal voltage-dependent closure of the unaffected channels. Secondly, it could also be hypothesized that aluminum binding to the channel does not impair the ability of the channel to close but the conductance of the resulting "closed" state is similar to that of the open state. Thirdly, aluminum may selectively stabilize the open state. Finally, aluminum may interact directly with the channel voltage sensor, thereby reducing the ability of the channel to sense and respond to an electric field.

We have rejected the first three possibilities for several compelling reasons. (i) The energy difference between the open and closed states is not noticeably changed by the presence of aluminum (*see* discussion below). This would not likely be the case if aluminum was binding to the channel to such an extent as to prevent closure or greatly stabilize the open state. (ii) The open and closed single channel conductances remain essentially unchanged by the addition of aluminum. (iii) Even in the presence of 100 μM aluminum, which virtually abolishes voltage-dependent closure at 50 mV, typical closure events are apparent. (iv) With the application of high enough voltages, multi-channel membrane conductance will decrease to typical, untreated levels, indicating that all the channels are able to achieve the normal low conducting state. (iv) The conductance of a membrane without channels is not changed by the addition of 100 μM aluminum chloride. Moreover, analysis of the voltage-dependence parameters: n, V_o, and nFV_o indicate that the aluminum entity interacts directly with the channel's voltage sensor.

Increased aluminum decreased the steepness of voltage dependence, n. This parameter, n, can be viewed as a measure of the minimal number of charges on the molecule that would have to respond to the electric field in order to account for the observed voltage dependence. The movement of these charges through the field causes a change in the conformation of the protein such that ion flow is reduced (i.e., the closed state). The electric field makes this altered protein conformation more stable, and, therefore, the probability that the protein will exist in the low conducting, closed state increases with the intensity of the electric field. Several lines of evidence indicate that the voltage sensor is composed of positively charged amino groups (Bowen et al., 1985; Doring & Colombini, 1985*a,b*). Therefore, the reduction in n, which occurs in the presence of aluminum, is consistent with the hypothesis that the negatively charged aluminate moieties neutralize the positive gating charges. This reduces the number of charges that can respond to the applied field, thereby reducing the probability that the channel will be in the low conducting state at a given applied voltage.

The increase in V_o and the constant nFV_o are consistent with this interpretation. A decrease in the number of charges that respond to the electric field reduces the energy available to stabilize the higher energy, closed state. V_o is the applied voltage at which half the channels are open. With a reduction in the number of gating charges, V_o increases to compensate for the energy lost through neutralization of gating charges. Finally, nFV_o, the energy difference between the open and closed states, is not affected by aluminum. This is expected since alteration of the gating charges alone

would alter only the probability of the channels being in either the open or closed state, not the inherent conformational differences between those states. In addition, since the net energy difference between the two states is unchanged, the increase in V_o is sufficient only to compensate for changes in the number of gating charges, n. Thus, the possibility that aluminum also significantly affects other regions of the protein is unlikely.

The effects reported here were observed following addition of $AlCl_3$ to buffered salts bathing the channel-containing membrane. The behavior of aluminum in aqueous solution is complex because of its six coordination sites and amphoteric nature. Meaningful interpretation of these findings require that we understand the nature of the active aluminum species. Knowing that aluminum is highly effective at pH 7 and ineffective at pH 4 seems to eliminate all positive forms of aluminum as candidates for the active form. The positive forms are either present in much higher concentrations at pH 4 (Table) or present at comparable concentrations at 4 and 7 pH levels. Thus the only contenders are aluminum hydroxide ($Al(OH)_3$) and aluminate ($Al(OH)_4^-$). Aluminate could simply neutralize the sensor as indicated in the preceding paragraph and therefore seems the candidate of choice. However, it is possible that the uncharged amino groups of the sensor chelate the aluminum hydroxide by displacing the three water molecules that coordinate the aluminum (of the six coordination positions, three are water and three are hydroxyl ions). This would shift the equilibrium of the amino groups toward the uncharged state, thus neutralizing the sensor. In either case the net effect would be a reduction in the charge on the sensor and, therefore, the ability to sense and respond to the field. Further studies are planned to determine the effective specie(s).

The results of these pH shift experiments could be interpreted differently. The observed restoration of voltage-dependent closure at pH 4 could be due to titration of a group(s) on the protein, which makes it less likely to bind aluminum. However, we believe this is unlikely since we observe little change in the properties of untreated VDAC channels at pH 4.

The effects of aluminum on the properties of the VDAC channel provide several new insights into its mode of action. Firstly, these data provide further evidence that the channel's voltage sensor is positively charged. Secondly, the findings indicate that the charges associated with the sensor are different from those that impart ion selectivity. If amino groups are responsible for both functions, as previously reported (Bowen et al., 1985; Doring & Colombini, 1985*a,b*; Adelsberger-Mangan & Colombini, 1987), then they may be organized differently, resulting in vastly different affinities for aluminum. $AlCl_3$ additions that virtually abolished voltage-dependent closure (100 μM) had no effect on either the channels preference for anions over cations or total channel conductance. Finally, this study indicates that, in contrast to the proposed model, the voltage sensor is most likely located outside the channel proper. If the sensor were located in the channel then the gating charge alterations resulting in loss of voltage dependence would certainly also be reflected in some change in either selectivity or total channel conductance. We see no such changes.

In summary, in the presence of micromolar levels of aluminum, the channel from the mitochondrial outer membrane, VDAC, is inhibited from undergoing voltage-gated closure. This effect is a useful probe for the voltage-gating mechanism in VDAC and may be involved in the process of aluminum toxicity.

This work was supported by NSF grant #DCB-85-10335 and ONR grant #N00014-85-K-0651.

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Received 30 April 1987; revised 15 July 1987