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Title of Dissertation: Probing the Nature of the Voltage-Sensing Mechanism of the Mitochondrial Outer Membrane Channel, VDAC: Initial Kinetic Analysis and Aluminum Chloride-Induced Alterations.

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PROBING THE NATURE OF THE VOLTAGE-SENSING MECHANISM OF THE MITOCHONDRIAL OUTER MEMBRANE CHANNEL, VDAC: INITIAL KINETIC ANALYSIS AND ALUMINUM CHLORIDE-INDUCED ALTERATIONS

by

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ABSTRACT

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Eleanor Thérèse Dill, SNDdeN, Doctor of Philosophy, 1987

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VDAC channels are the major permeability pathways through the mitochondrial outer membrane. They exist in a high conducting, "open", state at low potentials and a low conducting, "closed", state at high potentials (>+/- 20 mV). The mechanism underlying voltage-dependent behavior is poorly understood. VDAC isolated from Neurospora crassa were studied in planar phospholipid membranes. Aluminum chloride interaction with the protein and analysis of the rates of channel opening and closing were used to probe the mechanism of voltage-dependence. Micromolar amounts of aluminum chloride (>1 uM) decreased the steepness of the voltage-dependence and increased the voltage needed to close half the channels. Open and closed channel conductance levels were essentially unchanged. Neither channel conformation nor ion selectivity were altered. The effective aluminum species is either, or both, aluminate or aluminum hydroxide. The rate constants of channel opening and closing

were determined from multi-channel membrane studies. Closure rate constants increased exponentially with increased negative applied voltages, from 0.01/sec at -30 mV to 1.77/sec at -80 mV. Short periods (< 4-6 min) in the open state before closure decreased closure rates, indicting the presence of at least two open states. Opening rates were at least an order of magnitude faster than closure rates and had no marked voltage-dependence between -15 mV and -5 mV or +5 mV and +10 mV. However, the rate increased an order of magnitude between -5 mV and +5 mV. Channel closure accounts for 80%, or more, of the voltage-dependence observed in the steady state. Implications of these findings for modeling the action of the channel are discussed. As a result of these studies future models of the VDAC channel and research must consider these new complexities: distinct groups of charges are responsible for voltage-gating and selectivity; the sensor is likely outside the channel proper; channel opening may involve a large dipole; and opening and closing may occur via different molecular pathways.

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CHAPTER ONE: GENERAL INTRODUCTION

BACKGROUND

A dominant feature of cellular structure is the separation of cells and some organelles from their environment by membranes. This separation enables the existence of an environment inside the cell or organelle that facilitates its specific function(s). However, it is necessary for a large number of materials to enter and leave across this hydrophobic barrier. Membrane channels are one means of transmembrane transport. Channels are water-filled pores (usually formed by proteins) that span the membrane and permit the movement of water-soluble substrates and ions across the otherwise impermeable bilayer. Membrane channels exhibit selective permeability, being highly specific with regard to the materials that can pass through them. This specificity is broadly referred to as channel selectivity. In order to maintain the necessary internal environment and ensure proper function, the transport of materials across these pores is almost always highly regulated.

CHANNEL VOLTAGE-GATING

"Voltage-gating" is one way of controlling the permeability of a membrane channel. A channel is voltage-gated if the potential across the membrane determines the probability of the channel being in a high conducting, "open", or a lower conducting, "closed", state. The actions of a variety of

voltage-regulated channels are the basis for many critical physiological events including: nerve conduction, muscle excitation, cell-cell communication, egg fertilization, and insulin secretion. However, despite the critical roles played by voltage-gated channels, the actual mechanism underlying this voltage dependence is poorly understood. Clearly, the change from the open to the closed state occurs as the result of conformational changes in the protein. These states differ from each other by some inherent energy, and in the absence of any field the protein will assume the lowest energy conformation. The magnitude of the electric field alters the energy difference between the opposite forms such that a new equilibrium is established favoring one or the other state.

It is generally agreed (from first principles) that voltage-gating must result from the movement of charges on the protein relative to the transmembrane voltage or the alignment of large dipoles, in the protein, with the electric field. The charges or the dipole that respond to the electric field comprise the channel's voltage sensor. If the movement of this sensor were coupled to the opening or closing of the channel, then a voltage-gated channel would result.

Voltage-gating mechanisms can conveniently, though perhaps somewhat artificially, be considered in two categories. A number of channel formers enter the cell membrane from the exterior environment for example: alamethicin (Ehrenstein, Lacar and Nossal, 1970); DJ400B (Bohlmann, Dehmlow, Nuehahn, Brandt and

Bethke, 1970); monazomycin (Heyer, Muller and Finkelstein, 1976); colicin K (Schein, Kagan and Finkelstein, 1978); and erythrosin B (Colombini and Wu, 1981). These materials are essentially foreign to the membrane. The voltage-dependent membrane conductance seen in the presence of these channel-formers results from voltage-dependent insertion of the materials into the membrane. Once driven into the membrane they form channels of stable conductance. A mechanism for this type of voltage regulation has been proposed (Heyer et al. 1976; Eisenberg, Hall and Mead, 1973). Essentially, these materials are viewed as hydrophobic molecules having very strong dipoles along their long axes. They adsorb to the membrane surface with the long axis, and thus their dipole, parallel to the surface. In the presence of an electric field, the diploles align with the field and the molecules insert into the membrane. Interactions of the molecules with each other and the membrane during this process result in the formation of the channel.

In contrast to these "alien" channels, a large number of channel-formers are native protein components of the membranes; examples are: Na^+ channel (Hodgkin and Huxley, 1952a); amphibian gap junctions (Spray, Harris and Bennett, 1979); porin (Schindler and Rosenbusch, 1978) and VDAC (Schein, Colombini and Finkelstein, 1976; Colombini, 1980b). These channels are integral membrane proteins. The observed voltage dependence of conductance across membranes containing these channels seems more likely due to subtle changes in the conformation of the proteins

themselves in response to the electric field. The Na^+ channel has been studied extensively and a molecular model has been suggested (Cattarall, 1986) that illustrates the subtle nature of the molecular rearrangements that may be involved in voltage-gating. The primary structure of the molecule contains 4 unusual sequences that may form amphipathic helices and respond to the electric field by screwing a short distance into the membrane. Theoretically, this molecular twisting could result in the movement of +6 charges outward through the membrane. The movement of +6 charges was predicted by Hodgkin and Huxley (1952b) and demonstrated by Armstrong (1981). Indeed, Hodgkin and Huxley predicted the existence of 4 gating "particles".

THE VDAC CHANNEL

VDAC is a voltage-dependent channel located in the outer membrane of all mitochondria studied to date. Clues to the existence of a permeability pathway through the outer membrane were available from the functional and structural descriptions of the outer membrane as early as 1957 (Werkheiser and Bartley, 1957; Parsons, Bonner and Verboon, 1965; Parsons, Williams and Chance, 1966; Pfaff, Klingenberg, Ritt and Vogell, 1968; Wojtczak and Zaluska, 1969). However, the idea that large channels are responsible for the flux of metabolites across the outer membrane did not come into focus until these channels were discovered and reconstituted into phospholipid membranes. VDAC was first isolated from Paramecium and its properties in planar

phospholipid membranes described by Schein, Colombini and Finkelstein (1976). It exhibits a high conducting ,"open", state at low transmembrane potentials (< +/-20 mV) and a low conducting, "closed", state at high transmembrane potentials (> +/-20 mV) (Schein, et al., 1976). The open channel conductance is high (4.2 nS, nanoSiemens, in 1 M KCl) and the closed channel conductance is 40-50% of the open conductance (Colombini, 1980a, 1986).

Channel Structure The VDAC channel is composed of 1 or 2 (identical) polypeptides, having a molecular weight between 29 and 35 kDa depending on the source and methods used to estimate it (Zalman, Nikaido, and Kagawa, 1980; Linden, Gellefors, and Nelson, 1982a; Roos, Benz, and Brdiczka, 1982; Frietag, Neupert and Benz, 1982; Colombini, 1983; Nakashima, Mangan, and Pedersen, 1985). Studies of VDAC from rat liver mitochondria indicate that the functional channel is a dimer (Linden and Gellerfors, 1983; Colombini, 1980a; Roos et al., 1982). However, Mannella (1987) has recently suggested that evidence from image analysis of frozen-hydrated outer membranes of Neurospora crassa indicates that each channel is composed of a monomer - raising the intriguing possibility of significant structural differences between the mammalian and fungal channel.

Three dimensional reconstruction of micrographs of negatively stained N. crassa VDAC reveal that the channel is a simple cylinder through the membrane with no large aqueous domains (Mannella, Radermacher, and Frank, 1984). A large open-

state pore diameter, 2-4 nm, has been estimated by a variety of methods (Mannella and Ratkowski, 1979; Colombini, 1980b; Mannella, 1982).

Channel Selectivity Non-electrolyte movement through the channel is determined by size. The molecular weight exclusion limit is approximately 6 kDa. Thus, small molecules such as glucose can readily traverse the channel. However, the flux of ions and small electrolytes is charge dependent. Both cations and anions permeate the open channel, but it is weakly anion selective, with a preference for anions over cations of 2:1 (Cl^-:K^+) (Colombini, 1980b). In the closed state anion permeability is preferentially reduced.

There is no definitive picture of what aspect of the channel imparts its selectivity properties. However, since the channel pore diameter is large, even in the closed state, relative to alkali metal ion radii, it seems most probable that the weak anion preference results from positive charges near the mouth of the channel or lining the channel proper. The involvement of positive charges has been convincingly demonstrated using succinic anhydride, which replaces positively charged amino groups with negatively charged carboxyl groups (Doring and Colombini, 1983, 1985a; Adelsberger-Mangan and Colombini, 1987). Increasing concentrations of succinic anhydride decreased the preference of the channel for anions and, indeed, high concentrations reversed the channel's preference altogether to moderately cation selective.

Voltage-Gating The nature of the VDAC voltage-sensing mechanism has been studied most effectively with channels inserted into planar phospholipid membranes. Under these conditions, the behavior of the channels can be studied using voltage-clamp methods. In this way it is possible to control the voltage across the membrane and record the current through one, a few, or many VDAC channels.

There is strong evidence that the portion of the VDAC channel that actually detects the change in voltage across the membrane is a group of at least 5 positive charges, most likely lysine epsilon amino groups. Bowen, Tam, and Colombini (1985) demonstrated that the sensor was titratable. When the pH of the medium bathing the channels was increased from 6.2 to 10.7 the voltage dependence of the channels was reduced, such that higher voltages were required to close the channels. At the same time the energy difference between the open and closed states had not changed. Therefore, titration could reduce the number of effective charges that could respond to the electric field. Furthermore, succinic anhydride, which converts amino groups to carboxyl groups, eliminated VDAC voltage dependence (Doring and Colombini, 1985a). At physiological pH such treatment essentially changes the net positive charge to a net negative charge. Since the mean apparent pK of the titratable group was 10.6, arginine and lysine groups are likely candidates for the sensor. However, since the voltage dependence of the channel was unaffected by any of several arginine-modifying reagents (Bowen

et al.,1985) attention has focused on the lysine residues.

The initial descriptions of the voltage-dependent behavior of VDAC channel clearly demonstrated the presence of two switching regions, one at positive potentials and one at negative potentials (Schein et al. 1976). Later studies confirmed the presence of of two distinct closed states (Colombini, 1986). The effect of succinic anhydride on the voltage dependence of VDAC was used to further probe the location and nature of the voltage sensor (Doring and Colombini, 1985b). Channels were inserted into planar phospholipid bilayers and succinic anhydride was added to either or both sides of the membrane while the channels were held in either the open or closed state at either positive or negative voltages. Regardless of whether the channels were maintained in the open state at positive or negative potentials, anhydride addition to either side of a membrane caused a loss of voltage dependence. This indicated that the sensor was essentially equally accessible to the anhydride when in the open state at either potential. However, when channels were held in the closed conformation, a loss of voltage dependence occurred preferentially when the anhydride was added to the negative side of the membrane. These findings indicated that, in the closed state, the gating charges were more accessible from one side of the membrane, the negative side. This is exactly what one would expect if positive charges were to move in response to an applied potential. In addition, succinic anhydride modification of selectivity always went hand-in-hand with modification of voltage

dependence. These findings led to the development of a model in which the sensor is a single set of gating charges and the charges are located in the center of the channel.

A Model Of VDAC A simple model of the VDAC channel has been proposed (Doring and Colombini, 1985b). In this model the channel is viewed as a simple cylinder in the open state and as a cone in either the positively or negatively induced closed states. The two closed states result from the constriction of either of the two ends of the cylinder and the constricted region would always face the positive side of the membrane. The gating charges are a group of lysine amino groups located in the center (or at least within) the channel. (This same group of charges could stabilize either of the two closed states via alignment of the dipoles of the channel walls with the field and a reduction of the potential at the charges). This simple model proposes that the same group of charges are responsible for both VDAC's voltage dependence and anion selectivity. Although this is a unique proposal, it is consistent with the fact that both selectivity and voltage-gating are altered by the addition of succinic anhydride (as described above). Certainly, it is possible that 2 sets of charges each with different functions could both react with the same reagent. The behavior of the channel could also be modeled by 2 sets of gating charges, each of which can move entirely through the channel. However, in the absence of data to support such views, the simplest model had been constructed.

Physiological Relevance With so much attention focused on the respiratory function of the inner membrane and the matrix, mitochondrial research has paid little attention to the outer membrane. A wide range of substrates are required for and, result from, mitochondrial activity. Therefore, it seems clear that the outer membrane sits at the intersection of a tremendous amount of molecular traffic between the cytoplasm and the inner membrane space. Thus, it is reasonable to consider that the voltage-gated VDAC channel may play a major role in the regulation of mitochondrial activity. The protein is highly conserved and is present in the mitochondria of virtually every eukaryotic organism examined to date, including: mammals (Colombini, 1979), fungi (Colombini, 1980), protists (Schein et al., 1976), and higher plants (Smack and Colombini, 1985). VDAC comprises as much as 30 to 60% of the total outer membrane protein depending on the organism and in all these systems its properties are remarkably similar. Most convincing, Tedeschi, Mannella, and Boman (1987) recently succeeded in patch clamping the mitochondrial outer membranes of cuprizone-fed mice and observed voltage-dependent conductance changes similar to typical isolated VDAC voltage-gated behavior. In addition, the response of the patched-membrane conductance to the presence of agents, whose effects on isolated VDAC behavior are known, were essentially the same as those observed in isolated VDAC. There is also evidence that the VDAC protein may be the mitochondrial hexokinase binding site (Linden et al., 1982b; Nakashima et al., 1986).

Although more is known about the molecular mechanism underlying the voltage-gating of VDAC than of any other voltage-gated channel, there is still a long way to go. There is evidence that the voltage sensor consists of amino groups and that the location of these groups changes when the channel undergoes a conformational changes. However, the exact location of the charges, the nature of the conformational change, and the mode by which the charge movement is coupled to the conformational change are but a few of the questions that are unanswered.

While it seems highly likely that VDAC may play a significant physiological role, it is also an excellent model system for studying the basis of voltage-gating in transmembrane channels: 1) it is highly conductive (4.2 nS compared with 12 pS for the Na channel) and easy to study in both the open and closed states; 2) it has a stable open state that does not inactivate; 3) large amounts of the protein can be isolated, and purified if desired, from a wide range of organisms; 4) it has been sequenced and cloned from two species (Mihara and Sato, 1985; Forte, Guy and Mannella, 1986); 5) it has a low polypeptide molecular weight (about 30 kDa) and a large pore radius so that most of the protein must line the walls of the pore; 6) it just spans the membrane (Mannella, 1987) and thus, no large extra-membrane components need be considered; and finally, 7) the channels readily insert into planar phospholipid membranes, making both sides accessible to modification.

PRESENT WORK

Approaches In this thesis, I have probed the voltage-gating mechanism of the VDAC channel using two classic approaches: kinetic analysis and interaction of the channel with chemical agents. Since voltage-gating is indeed a series of molecular events, the properties of the channel as it moves through those events are rich with information about the gating mechanism that steady-state data does not contain. The properties of the channel, when interacting with known reagents, can contain diverse information ranging from identifying reactive sites to effects of blocking reactive sites.

The altered conductance through a channel as the result of an applied electric field derives from conformational changes in the protein. The rate with which the channel assumes any given state reflects the time required for the many necessary molecular rearrangements to occur. Therefore, the time required to assume another state will depend on the magnitude of the energy needed to accomplish the necessary molecular reordering. Thus the rate constant of the process embodies information concerning these rearrangements.

Kinetic analysis and reagent interaction studies, individually and in concert, have been powerful tools for probing the voltage-gating mechanism of diverse types of channels. A few examples will serve to illustrate. The voltage-dependent nature of the gap junctions of amphibian blastomeres was established

through elementary kinetic studies (Harris, Spray, and Bennett, 1981). The time constants of the conductance change from the high to the low conducting state were a linear function of the transjunctional voltage. The calculated rate constants for closure were twice as voltage-dependent as those for opening. Also, at least some of the channels enter the open state when being closed from one voltage to the same voltage of opposite polarity. Based on these early findings and structural data about the junction, a two gate, four state model was put forth. There are 2 "hemichannels" each with a set of gating charges responsive to the local voltage. A dipole is postulated to account for the difference in the rate constants of opening and closing. When in the closed conformation the component of the dipole normal to the field is reduced, therefore, the energy contributed to a closed hemichannel by the field is less than that to an open channel.

In the original descriptions of sodium channel behavior, Hodgkin and Huxley (1952b) described the activation, inactivation and closing of the channel. They proposed a model based on the suggestion that both an activation and an inactivation "gate" existed. However, the existence of two distinct gates was conclusively demonstrated 21 years later by Armstrong, Bezanilla, and Rojas (1973). They showed that the inactivation gate could be eliminated by pronase treatment such that inactivation events did not occur. The rate constants of channel opening before and after the pronase treatment were determined and compared. In the

absence of inactivation, activation kinetics were unchanged. Thus, the activation and inactivation processes are distinct and involve distinct gating mechanisms. In addition, since it had been shown that activation kinetics were unchanged by pronase, the activation mechanism could now be studied in isolation.

Analysis of the kinetics of single channel opening during depolarization revealed that, in at least mammalian cells, the sodium current has a fast and a slow component and that the slow component is the rate limiting step in the decline of the sodium conductance (Aldrich, Cory, and Stevens, 1983). Furthermore, comparisons of the rate constants of activation and inactivation over the range of depolarizing voltages showed that the inactivation process is essentially voltage independent.

As described earlier the modification of the VDAC channel with succinic anhydride revealed the probable nature of its sensor: lysine amino groups. In addition, careful observation of the channels in transition between states has made it possible to eliminate voltage-dependent membrane constriction as the source of the voltage-dependent behavior (Colombini, 1986). When the channels are closed with a negative transmembrane potential and the potential is then switched to a positive potential of equal magnitude, the channels instantaneously reopen and close again. Since constriction of the membrane is a function of the magnitude of the field, the return to an open state indicates that the channel is itself capable of conformational changes independent of membrane state.

The Planar Bilayer System These studies have been conducted with the VDAC channels reconstituted into planar phospholipid bilayers. The bilayers are formed by the apposition of two monolayers of a known lipid over a small (diameter = 0.15 mm) hole in a Saran partition. This partition separates two chambers each containing about 5 ml of solution. The aqueous environment of the channels can be very accurately maintained and adjusted separately on either side when necessary. Similarly, the lipid environment can be altered by the choice of the lipid with which to form the bilayer. A pair of calomel electrodes interface the two sides of the chamber with voltage-clamp instrumentation. Thus, the voltage across the channel-bearing membrane can be carefully controlled. The change in current that is detected across the membrane is due essentially only to the changes in the conductance of the VDAC channels under any given set of circumstances. Consequently, it is possible to study the behavior of the channel in isolation from a host of other cellular events and membrane proteins.

Certainly, the advantages of such a system are tremendous. It is possible to observe the behavior of single channels in the membrane as well as that of hundreds and to correlate the channel behavior with known events. However powerful the planar phospholipid bilayer system is for probing channel function, it is not without disadvantages. Certainly, the isolation of the channels that it provides and the controlled environment in which

observations are made would lead some to question the "biological relevance" of the observations. Supporting information obtained from less-altered environments (patch-clamping of the outer membrane, whole-mitochondrial studies, and whole-cell studies) is important. However, the detailed information sought here is difficult or impossible to obtain in an intact system. Only when the predictions and insights gained from these studies are applied to the physiological situation of interest, will the physiological relevance of the findings become clear.

In this thesis I have sought to further elucidate the voltage-gating mechanism of the mitochondrial outer membrane channel VDAC. I have conducted an initial analysis of the rates of the VDAC channel closure and opening and have identified and analyzed an aluminum-induced inhibition of the voltage-dependent behavior.

CHAPTER TWO: Voltage Gating in VDAC is Markedly Inhibited by Micromolar Quantities of Aluminum

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, Klein and Mayor, 1985; brain: Perl, 1985; parathyroid gland: Mayor and Burnatowska-Hledin, 1983) and disrupt a broad spectrum of cellular processes including membrane transport systems (Ca^{+2}: Siegel and Haug, 1983, Burnatowska-Hledin and Mayor, 1984; phosphate: Dousa and Kempson, 1982, Burnatowska-Hledin, Klein and Mayor, 1985; choline uptake and Na-K-ATPase activity: Lai et al., 1980) and enzymatic activity (bone phosphatases: Lieberherr et al., 1982; brain cytosolic and mitochondrial hexokinases: Solheim and Fromm, 1980, Lai and 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 and Wyatt, 1983; Perl, 1985), amyotrophic lateral sclerosis and parkinsonism-dementia (Klatzo et al., 1965; Terry and Pena, 1965; Perl and Brody 1980a,b; Perl et al., 1982; Garruto et al., 1984). The osteo-malacia, anemia, and dementia often associated with

long-term hemodialysis have also been linked to aluminum toxicity (Parkinson et al., 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 and Fromm, 1980; Lai and 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 et al., 1980; Neet et al., 1982). Finally, aluminum depresses mitochondrial respiration (state 3) and increases mitochondrial permeability (Burnatowska-Hledin, Ebner and Mayor, 1985).

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 et al., 1966; Mannella and Bonner, 1975; Schein et al., 1976; Colombini, 1979; Zalman et al., 1980; Freitag et al., 1982; Linden et al., 1982; Roos et al., 1982; Mannella and Colombini, 1984: Nakashima et al., 1986; Smack and Colombini, 1986). Its major characteristic properties are: 1) the aqueous pathway formed by the channel is large (1.5 to 2 nm in radius, Colombini, 1980b; Mannella, 1982) and highly

conductive (4.2 nS in 1 M KCl); 2) 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, 1980a,b); 3) the channel is weakly anion selective (Schein, et al., 1976).

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 (Doring and Colombini, 1985a,b; Bowen et al., 1985; 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 I 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 hypoosmotically 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 the 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 and 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 stepwise 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, $\hat{\tau}$, 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.

Single channel conductance levels were obtained from recorded step-wise changes in membrane current. These discrete current changes were quantified and converted to conductance values using a Hewlett-Packard digitizer and HP-85 computer. The open-state conductances were obtained from the discrete current increases which occur when single channels insert into a membrane. The closed-state conductances were obtained by subtracting the conductance changes upon voltage-induced channel closure, from the open-state conductances.

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 et al. (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:

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

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 no energy difference exists 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 voltage-axis intercept of the plot of $\ln (G-G_{min}/G_{max}-G)$ vs. Voltage respectively.

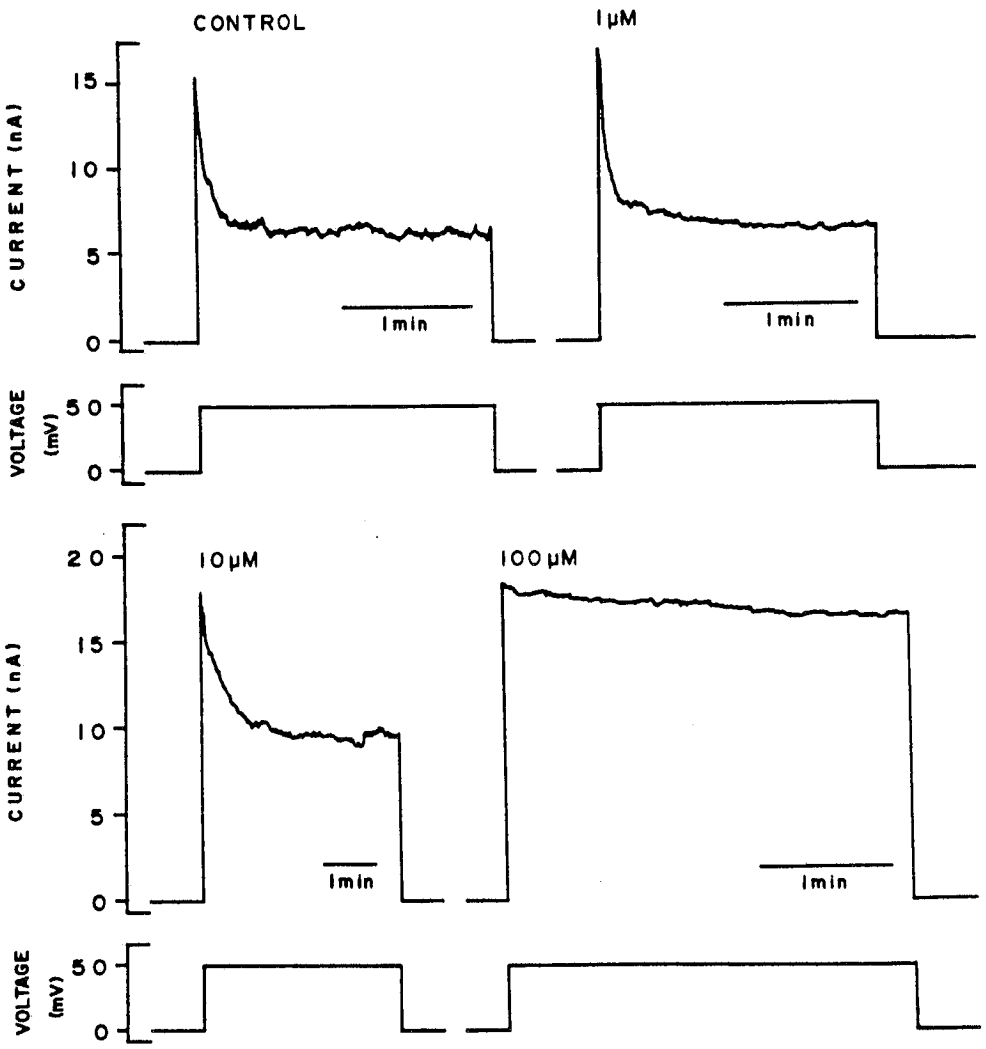
Obtaining correct estimates of n and V_o, therefore, depends on having accurate G_{max} and G_{min} values. In these experiments it was difficult to obtain G_{min} values directly in all situations because with the addition of $AlCl_3$ a larger voltage must be applied to achieve G_{min} (see RESULTS). These high voltages were prohibitive because they cause membrane breakdown. However, experiments with only a few channels in the membrane indicated that with 100 uM added $AlCl_3$, the mean open and closed channel conductances were not substantially different from those observed in $AlCl_3$-free controls (see RESULTS). 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. While there may be some decrease in the closed channel conductance in the presence of aluminum, I 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.

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 were normalized relative to it.

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 increase the time

Figure 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 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.


CONTROL
1μM
CURRENT (nA)
15
10
5
0
1min
1min
VOLTAGE (mV)
50
0
10μM
100μM
CURRENT (nA)
20
15
10
5
0
1min
1min
VOLTAGE (mV)
50
0

required to achieve a steady state closed channel 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. Therefore, I examined the open and closed state conductance properties of single channels in the presence and absence of $AlCl_3$ (100 μM). Panel A of Figure 3 a shows a current record obtained with a 9-channel membrane in the absence of added aluminum (from the membrane conductance and from observed channel insertions, the number of channels in the membrane was known). The application of only a -30 mV potential (at the arrow) results in the stepwise decrease in membrane conductance interpreted as the closure of individual channels. Four channels close within less than 1 min. Channels (though not necessarily the same ones) continue to open and close but the probability of occupying the open state is greatly reduced even at -30 mV. The step-wise increases and decreases in current that follow reflect the probabilistic opening and closing of the channels. The channel behavior illustrated in Panel A is typical of channel responses to low, closure-inducing potentials. In the presence of aluminum higher potential are required to elicit similar single channel responses. Panel B of Figure 3 illustrates the effect of 100 uM aluminum on single-channel behavior. Current records are shown of a 4-channel membrane (not that in Panel A) in the presence of

Figure 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 Figure 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.

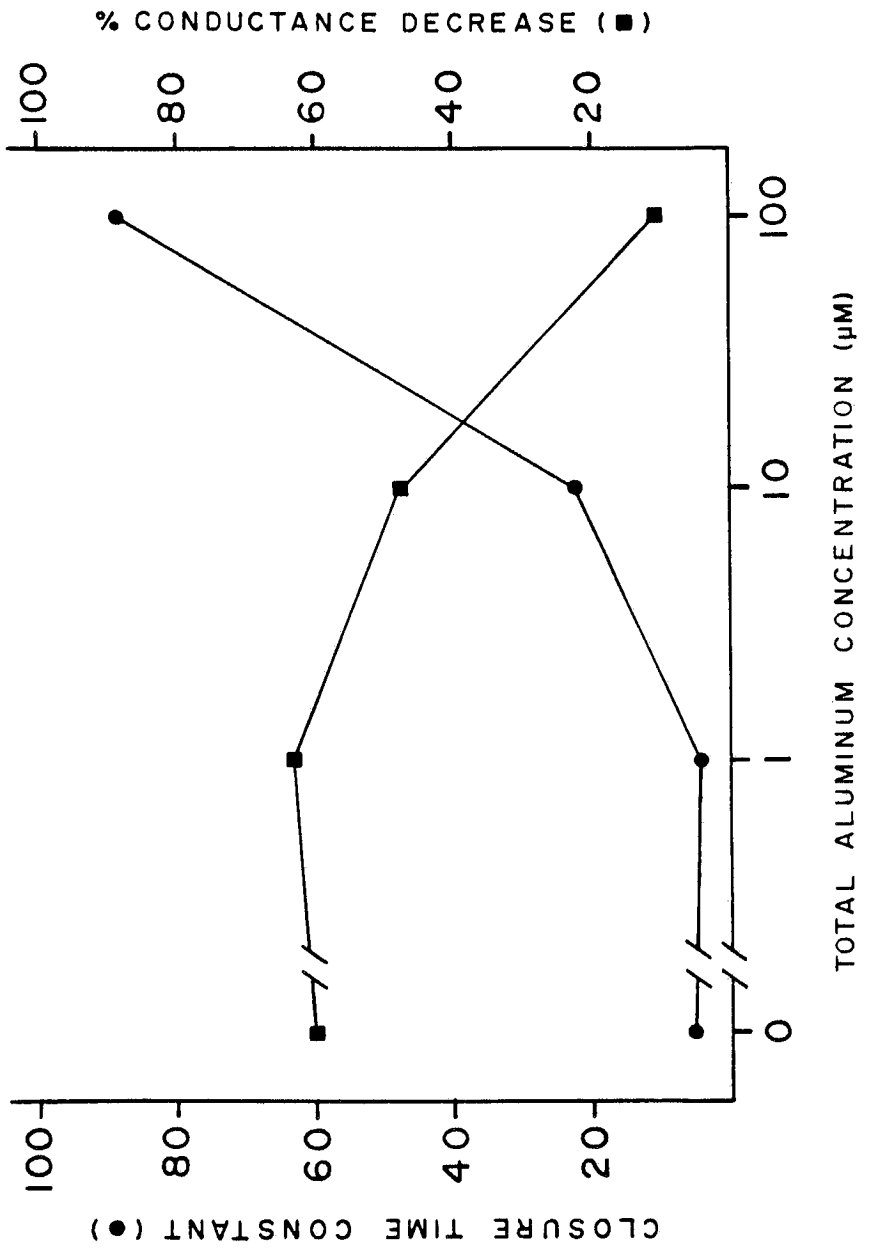
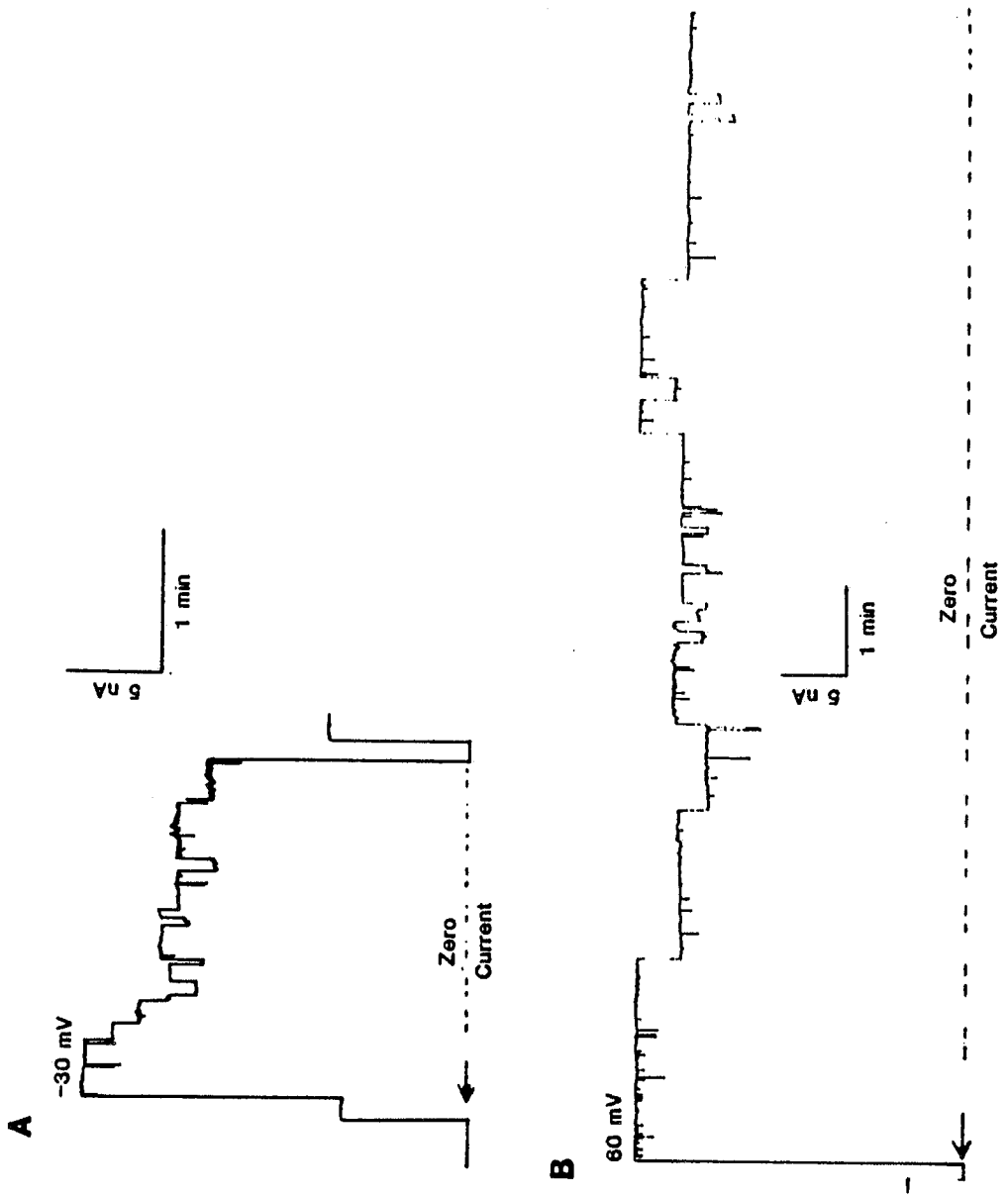
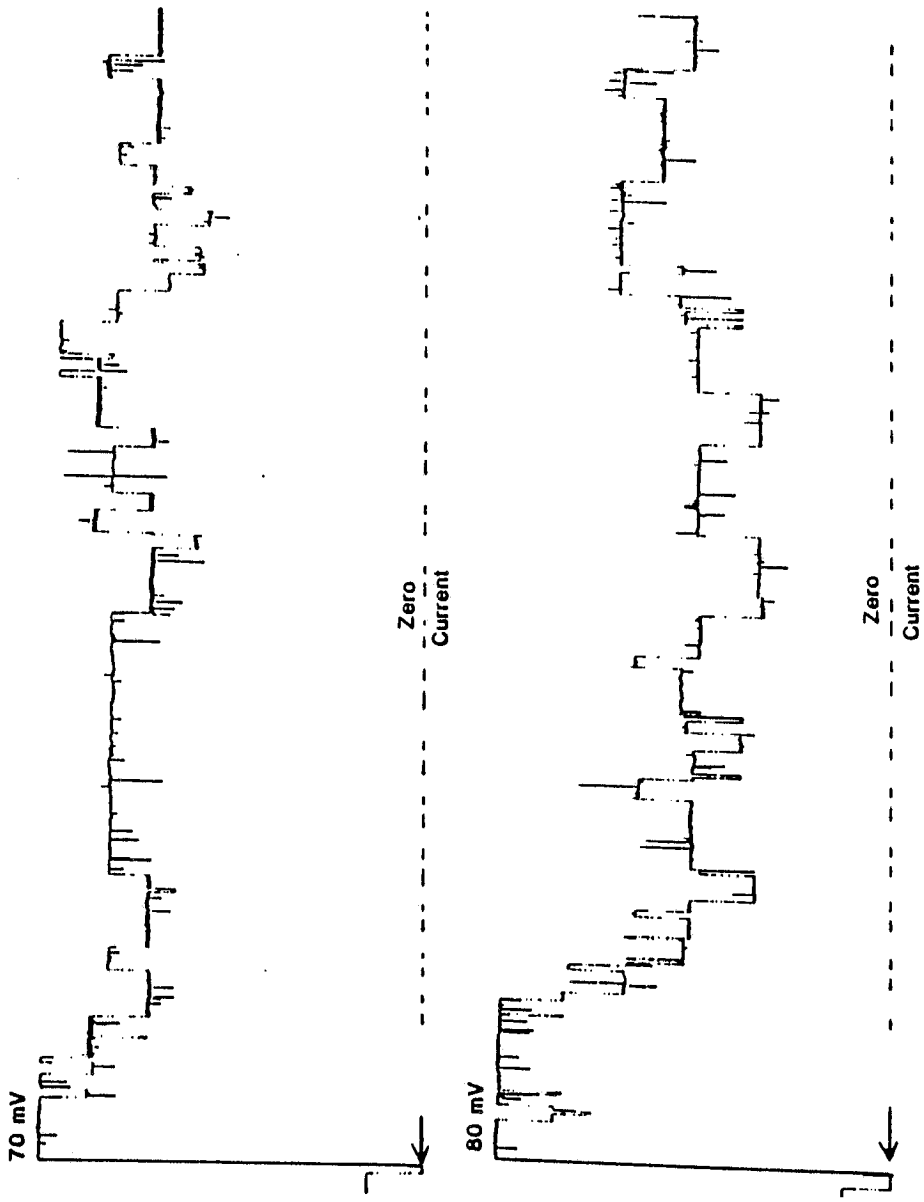

% CONDUCTANCE DECREASE (■)
100
80
60
40
20
100
10
1
0
TOTAL ALUMINUM CONCENTRATION (μM)
CLOSURE TIME CONSTANT (●)
100
80
60
40
20

Figure 3. VDAC channels can close in the presence of aluminum chloride but require higher transmembrane potentials to do so. Panel A is the current record of a 9-channel membrane in the absence of aluminum chloride. At the arrow the transmembrane potential was stepped to -30 mV. At this low potential the closed state is favored in the absence of aluminum. Panel B contains current records of a 4-channel membrane treated with 100 uM aluminum chloride. The records are of the same membrane exposed to increased transmembrane potentials of 60, 70 and 80 mV respectively. Higher potentials increase the probability of the channels being in the closed state. All other experimental conditions as in Figure 1.


A
-30 mV
5 nA
1 min
Zero
Current
B
60 mV
5 nA
1 min
Zero
Current

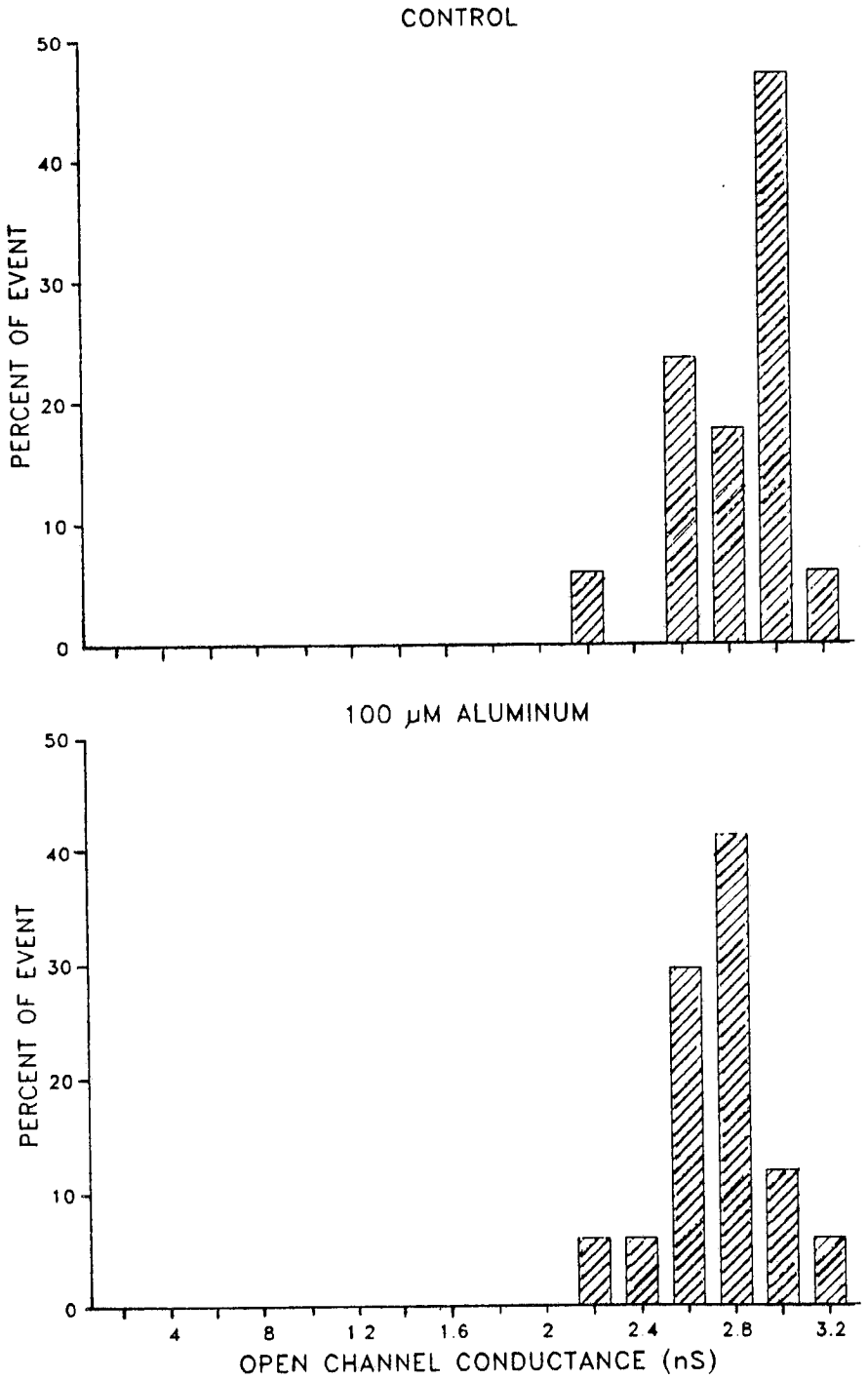

70 mV
Zero
Current
80 mV
Zero
Current

a 60, 70 and 80 mV potential. Note that the application of 60 mV causes only two channels to close and, although it is clear that the channels can close, the probability of the channels being closed is low. The probability of the channels being in the closed state increases when the transmembrane potential is raised to 70 and 80 mV. The differences in the single-channel current changes observed with increasing transmembrane potential result from the increased driving force. Thus, the qualitative picture is consistent with aluminum inducing a reduction in the probability of channel closure (at a given potential) rather than a change in channel conductance.

A quantitative analysis supports the above conclusion. Open channel conductance values were quantified from the individual, step-wise current increases evident during channel insertion in the presence and absence of aluminum. Histograms of these events (Fig. 4) show no significant effects of the addition of 100 μM aluminum. The mean open channel conductance was 2.8 nS (S.D.= +/-0.3 nS) for both with and without aluminum.

Closed channel conductances were determined from current recordings such as those shown in Figure 3. Membranes containing few channels were used in order to resolve the events and measure them accurately. The discrete drops in conductance observed at high potentials are the change in open-channel conductance which occurs when a channel closes. Therefore, in order to estimate the closed-state conductances, the changes in open channel conductance were subtracted from the mean open channel

Figure 4. Histogram of the open conductance of VDAC channels in the absence and presence of 100 μM aluminum chloride. Open channel conductance values were calculated from the step-wise current increases observed during channel insertion at -10 mV, a potential at which the channels are open. Channels were inserted in the presence and absence of 100 μM aluminum chloride. Control distribution is from 35 insertions which occurred in 4 experiments. Aluminum treated values were obtained from 35 insertion events in 6 experiments. All other conditions as in Figure 1.


CONTROL
PERCENT OF EVENT
50
40
30
20
10
0
100 μM ALUMINUM
PERCENT OF EVENT
50
40
30
20
10
0
4
8
1.2
1.6
2
2.4
2.8
3.2
OPEN CHANNEL CONDUCTANCE (nS)

conductance. Channel closure occurred less frequently in the presence of aluminum. However, it could be observed especially at higher potentials (60-80 mV). Histograms of the closed channel conductances are changed by the addition of 100 μM aluminum (Fig. 5). In 100 μM aluminum there was a marked broadening of the range of closed-conductance states assumed by VDAC channels and an obvious shift toward lower conductance states (FIG. 5). However, the mean closed channel conductance was not significantly increased by aluminum (control, 1.0 +/- 0.3 nS; aluminum, 0.8 +/- 0.5 nS). Although the shift in the histogram is observed in the presence of aluminum, other factors, such as the high applied voltages or altered closed channel selectivity, may be responsible. Most significantly though, the shift to lower conducting states cannot account for the observed increased membrane conductance in the presence of aluminum since the shift is in the direction of enhanced closure.

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 Figure 6. 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. 6, A and B). Thus, aluminum does not alter VDAC's mean closed state conductance, but reduces the probability that the channel will be in that closed state at a normal closure voltage.

Figure 5. Histogram of the calculated closed conductance of VDAC channels in the absence and presence of 100 μM aluminum chloride. Conductance changes were calculated from the discrete current drops which occurred with the application of a closure-inducing potential, 60 - 80 mV. The conductance drop was subtracted from the mean open channel conductance (2.8 nS) to give the closed channel conductance. Control values obtained from 89 closure events of approximately 35 channels in 4 experiments. Aluminum treated values were obtained from 240 events from 4 experiments. All other conditions as in Figure 1.

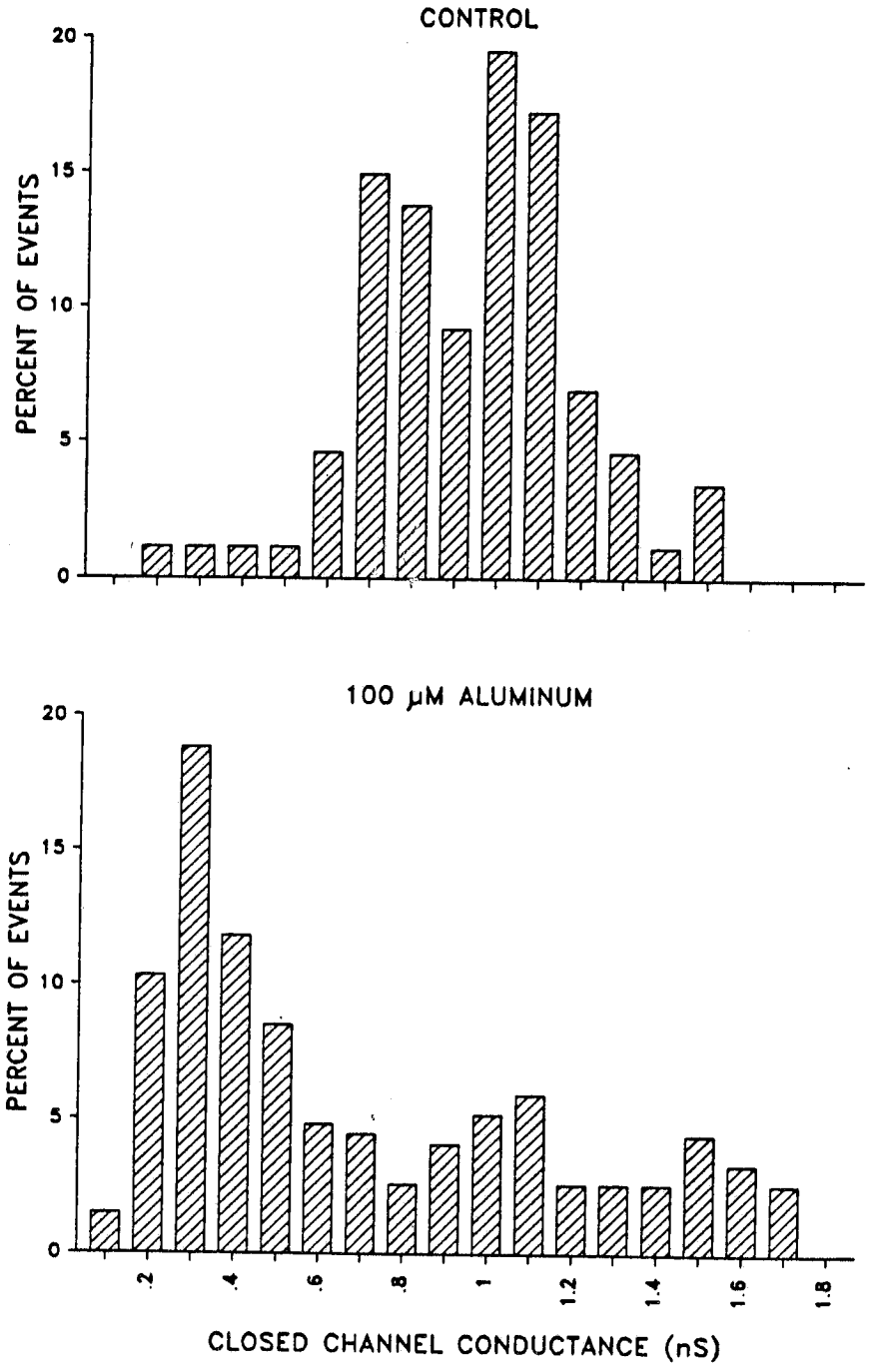
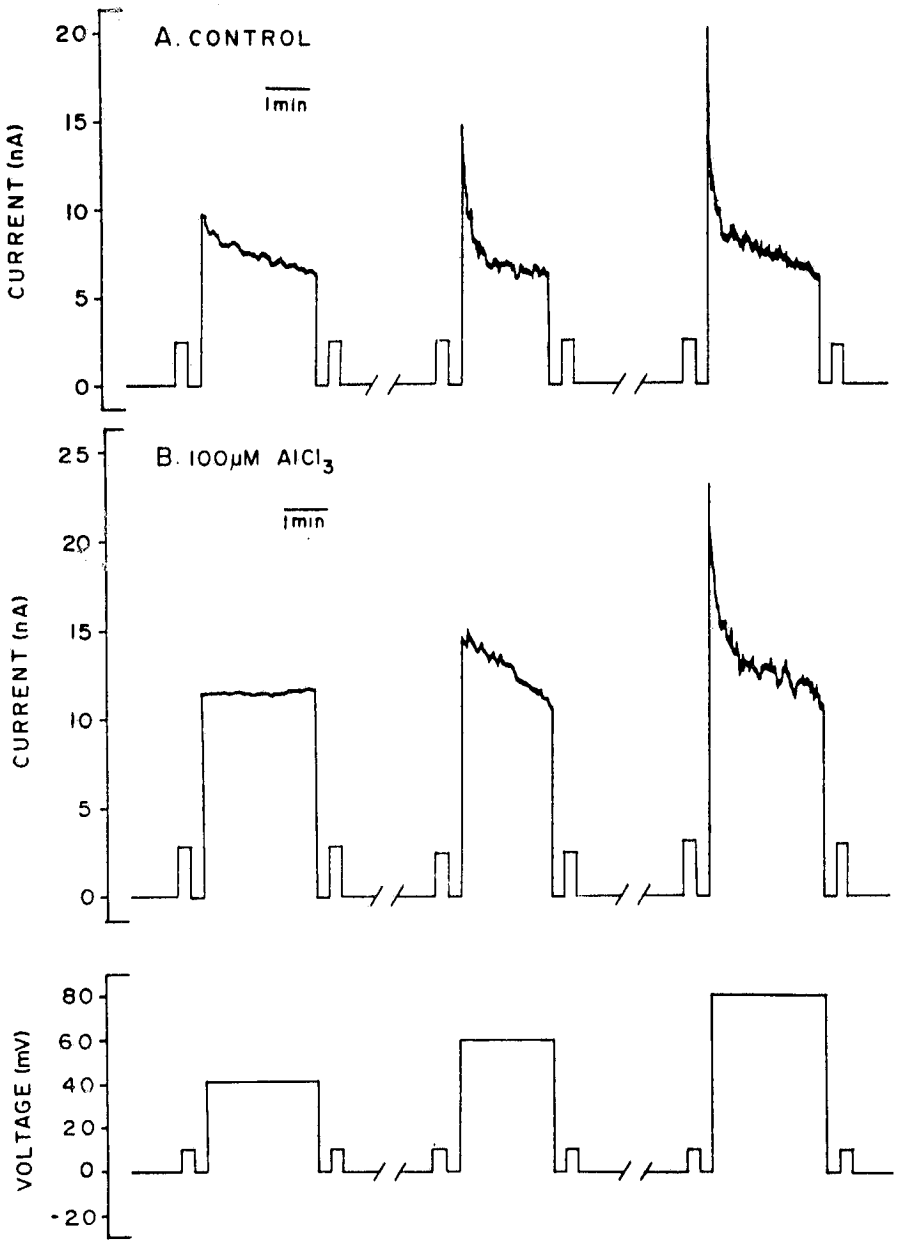

CONTROL
PERCENT OF EVENTS
20
15
10
5
0
100 μM ALUMINUM
PERCENT OF EVENTS
20
15
10
5
0
.2
.4
.6
.8
1
1.2
1.4
1.6
1.8
CLOSED CHANNEL CONDUCTANCE (nS)

Figure 6. Aluminum chloride treatment increases the voltage needed to close the channels. Record of the current through the same multi-channel membrane in the presence and absence of 100 μM aluminum chloride at closure-inducing potentials. 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). All other conditions as in Figure 1.


A. CONTROL
1min
CURRENT (nA)
20
15
10
5
0
B. 100μM AlCl3
1min
CURRENT (nA)
25
20
15
10
5
0
VOLTAGE (mV)
80
60
40
20
0
-20

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 mV 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 Figure 7 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 I 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. 7) were fitted to equation 1. Samples of transformed results are shown in Figure 8. $AlCl_3$ additions decreased the steepness of the voltage dependence, n, and increased the voltage required to close half the channels, V_o (Fig. 9). 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

Figure 7. Aluminum decreases the voltage dependence of VDAC's steady state conductance. Triangular voltage waves from 65 to -65 mV were applied (25 mV/sec) across multi-channel membranes. The current recorded as the voltage decreased was converted to conductance, normalized to the conductance at zero voltage and plotted. Controls, no $AlCl_3$ (X) and 100 μM final $AlCl_3$ concentration (□). All other conditions as in Figure 1.

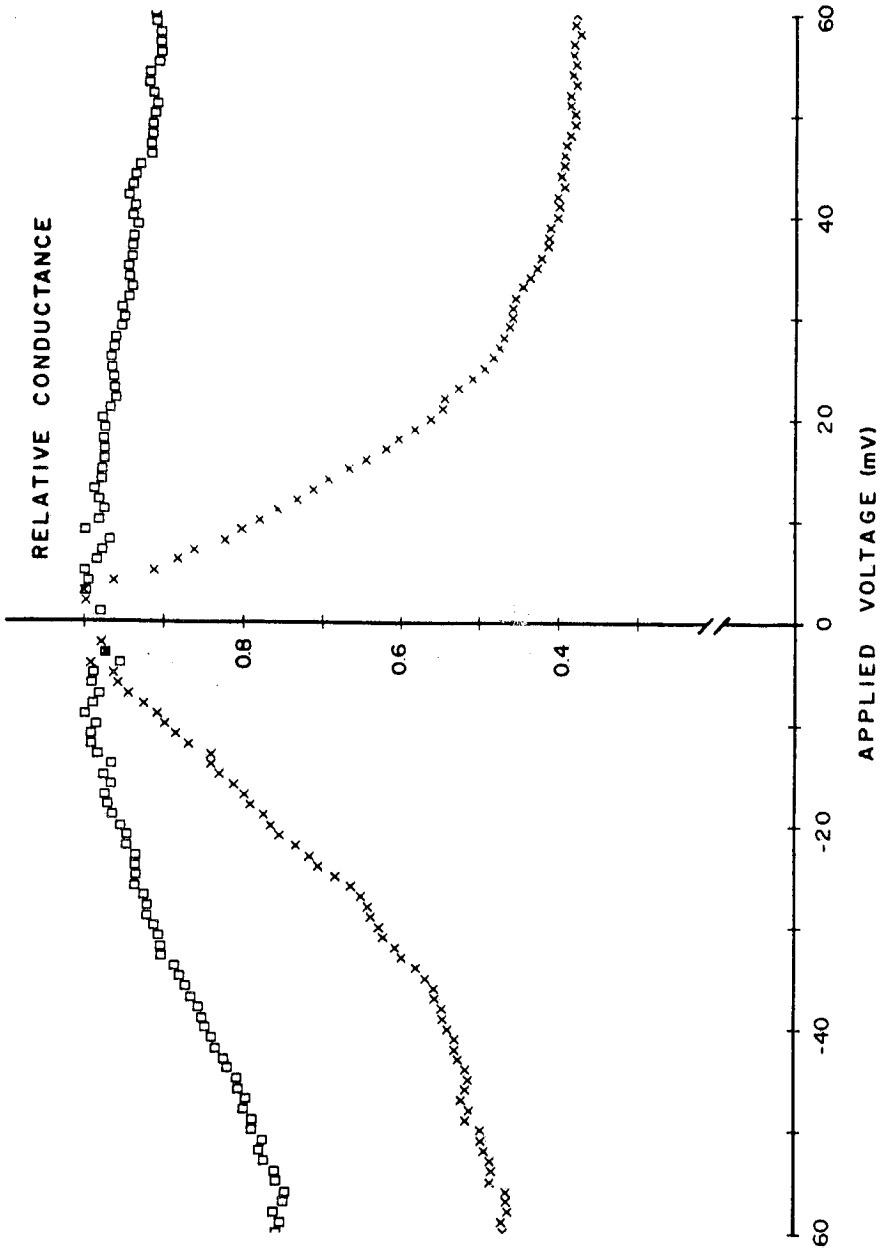

RELATIVE CONDUCTANCE
0.8
0.6
0.4
0
20
40
60
-20
-40
-60
APPLIED VOLTAGE (mV)

Figure 8. Linearized membrane-conductance data of a typical experiment. Each conductance point was log-transformed as in equation 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 equation 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 $AlCl_3$(X); n=3.0, V_o=13 mV: 10 μM $AlCl_3$(Δ); n=1.5, V_o=57 mV: 100 μM $AlCl_3$(+); n=0.9, V_θ=100 mV.

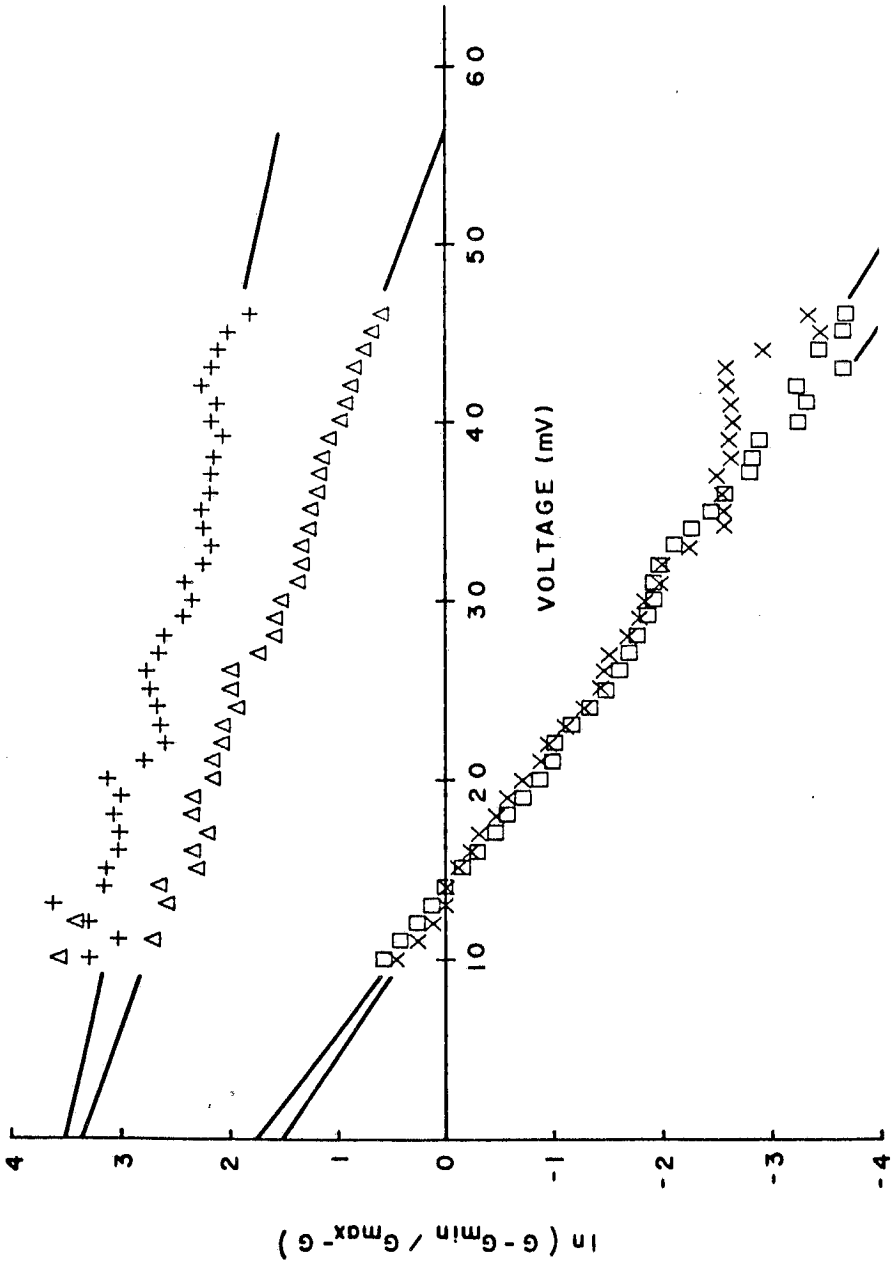
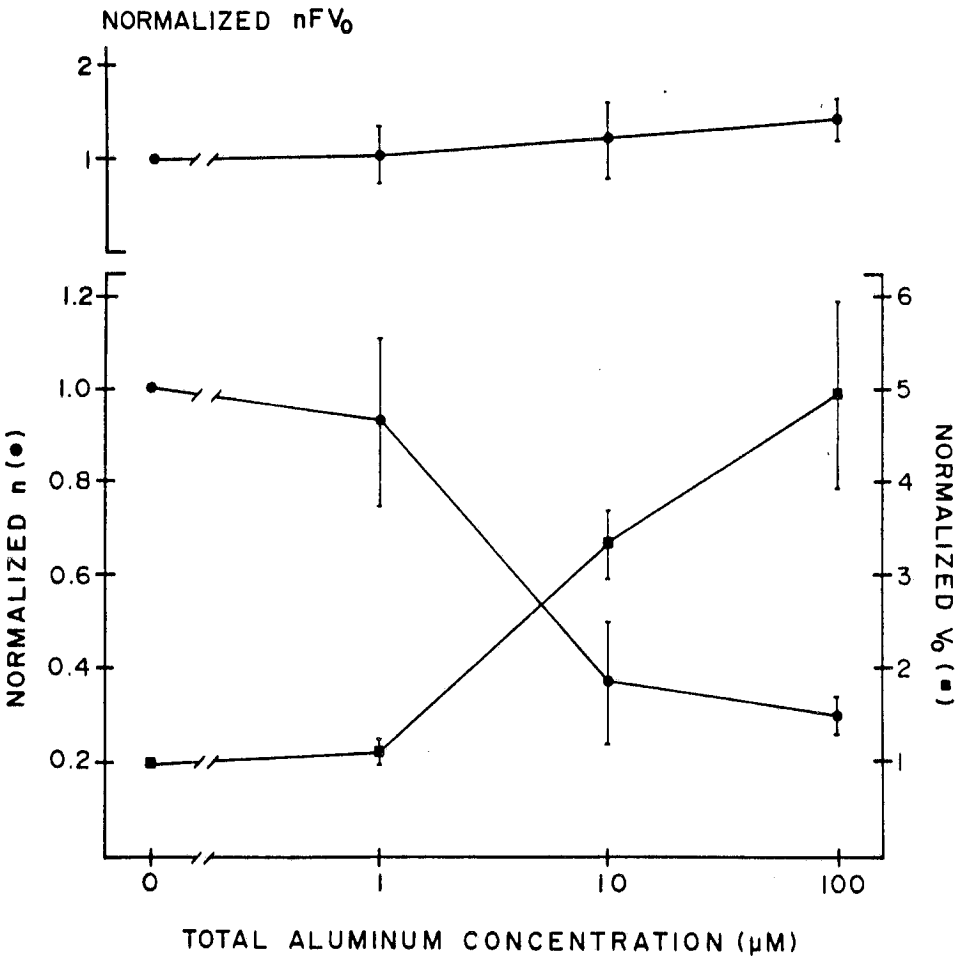

ln (G - Gmin / Gmax - G)
VOLTAGE (mV)
4
3
2
1
0
-1
-2
-3
-4
10
20
30
40
50
60

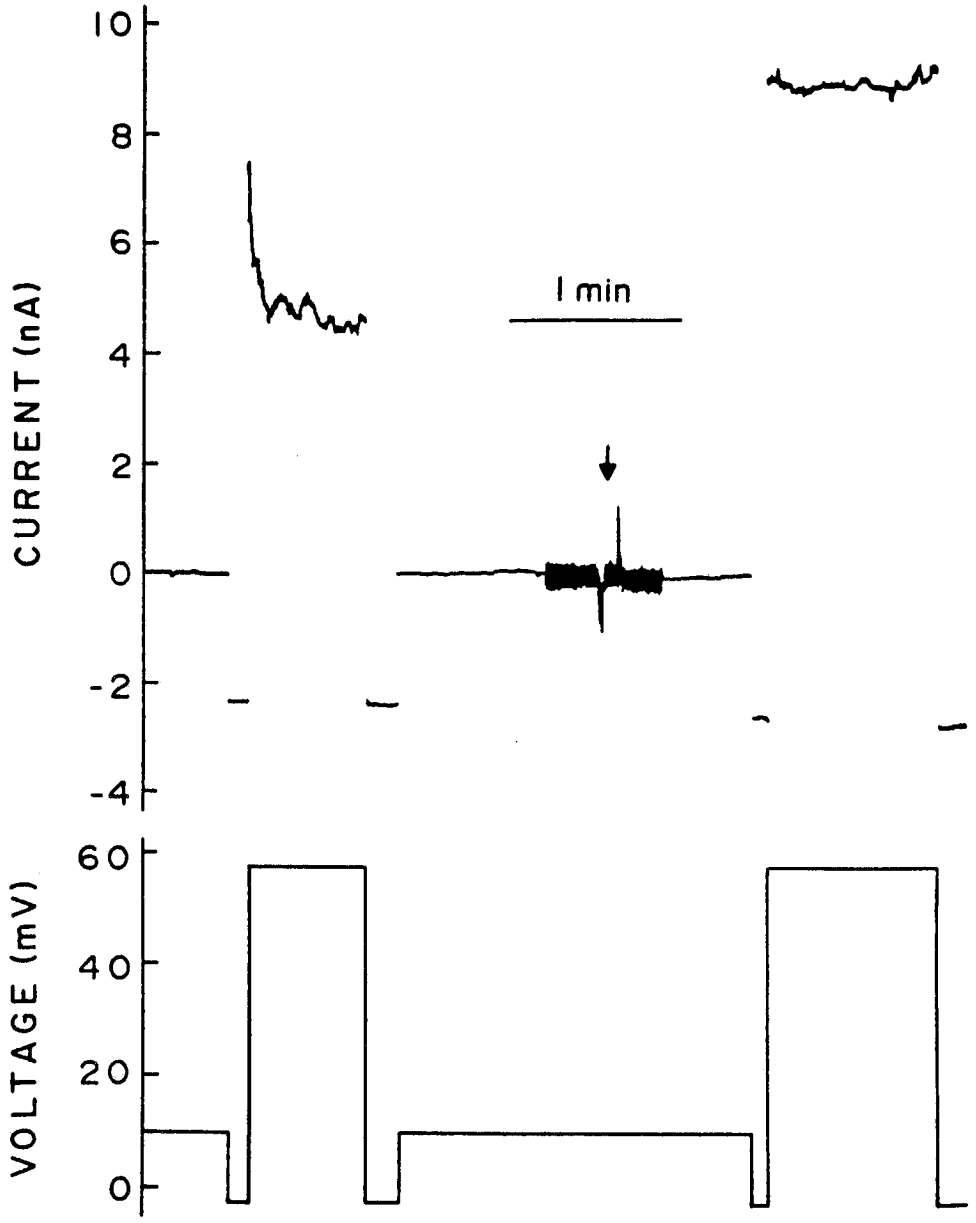
Figure 9. Aluminum changes the voltage-dependence parameters of VDAC. Plots, such as those shown in Figure 5, were generated for several experiments and analyzed (see MATERIALS and METHODS). With increasing aluminum concentration, the steepness of voltage-dependence, n (●) decreases; the voltage at which 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.


NORMALIZED nFV0
2
1
1.2
1.0
0.8
0.6
0.4
0.2
NORMALIZED n (●)
6
5
4
3
2
1
NORMALIZED V0 (■)
0
1
10
100
TOTAL ALUMINUM CONCENTRATION (μM)

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. 9). 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 was 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 multi-channel membrane formed across a 10-fold salt gradient was monitored. A potential was then applied to bring the current to zero (Fig. 10). 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, the reversal potential was determined. Again an attempt was made to close the channels by applying 50 mV.

Figure 10. Aluminum does not effect 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 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 concentration) 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.


10
8
6
4
2
0
-2
-4
CURRENT (nA)
1 min
60
40
20
0
VOLTAGE (mV)

The reversal potential of unmodified channels was 10 mV and was not changed by the addition of 100 μM $AlCl_3$ (Fig. 10). Furthermore, Figure 10 illustrates that while the addition of $AlCl_3$ caused the typical loss of voltage-dependent closure, ion selectivity remained unchanged.

Effective Aluminum Species. 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 species present in the chamber at a given pH was calculated (Table 1). 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 hydroxo-aluminum 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.

Table 1. Aluminum Species in Solution*

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

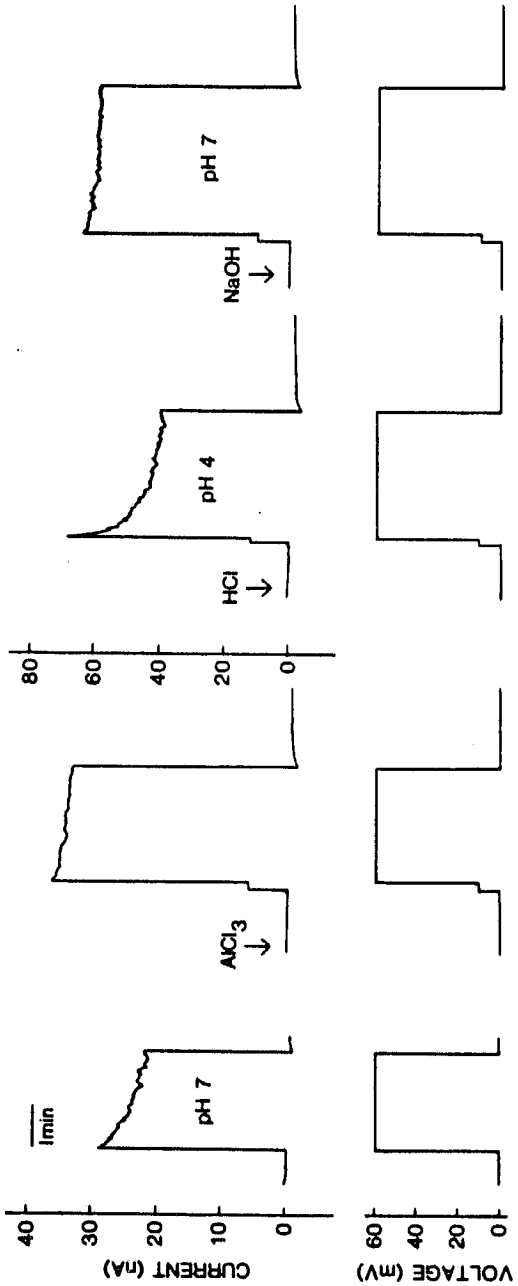
* calculated from dissociation constants (20°C, 0 ionic strength) obtained from Smith et al., 1976.

$AlCl_3$-induced reductions in voltage-dependent closure were routinely observed at pH values between 5 and 7. Figure 11 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.

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 formulate a model (Doring and Colombini, 1985b; 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 which results in preferential anion flow. The model has been consistent with subsequent work. Increasing the pH of the

Figure 11. 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 current 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.


CURRENT (nA)
VOLTAGE (mV)
1min
pH 7
AlCl3
HCl
pH 4
NaOH
pH 7

bathing solution reduced the steepness of voltage dependence, again indicating the presence of positive gating charges (Bowen et al., 1985). Furthermore, the pKa values of the titratable groups suggests that the charges are lysine epsilon amino groups. Most recently, Adelsberger-Mangan and Colombini (1987) used succinic anhydride to convert the proposed positive charges to negative charges (Adelsberger-Mangan and 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.

I have rejected the first three possibilities for several compelling reasons. 1) 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. 2) The mean open and closed single channel conductances remain essentially unchanged by the addition of aluminum. 3) Even in the presence of 100 uM aluminum, which virtually abolishes voltage-dependent closure at 50 mV, typical closure events are apparent. 4) 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. 5) 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 and 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 observed increase in the range of closed-conductance states in the presence of aluminum as well as the apparent shift toward lower conductance closed states suggests several possibilities. First, it is possible that the changes are the result of the high voltages required to close the channels in the presence of aluminum. Since these low-conductance states do occur in channels closed at lower potentials they may represent highly relaxed closed states. The higher closure-inducing potentials required in the presence of aluminum may increase the probability of the channels assuming this state. Secondly, these changes may reflect altered closed channel selectivity. VDAC's ion selectivity changes markedly upon channel closure. A preferential reduction in anion permeability of 70-90% accompanies VDAC closure (depending on the "closed state"

achieved by the channel) (Colombini,1980b). No systematic studies have been reported on the effect of high applied potentials on either the closed states of the channel or the selectivity of the closed states. In view of the significant effect of aluminum on the voltage-gating of the VDAC channel, studies on the possible effects of aluminum on the closed states achieved by the channels, should be pursued.

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 6 coordination sites and amphoteric nature. Meaningful interpretation of these findings requires an understanding of the nature of the active aluminum species. Knowing that aluminum is highly effective at pH 7 and ineffective at pH 4, eliminates 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 1) 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 species.

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 make it less likely to bind aluminum. However, this is unlikely since I 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 which impart ion selectivity. If amino groups are responsible for both functions, as previously reported (Bowen et al., 1985; Doring and Colombini, 1985 a,b; Adelsberger-Mangan and Colombini, 1987) then they may be organized differently, resulting in vastly different affinities for aluminum. $AlCl_3$ additions which 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. No such changes are observed.

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.

CHAPTER THREE: Kinetic Analysis of the Opening and Closing of the VDAC Channel

Transmembrane channels (usually proteins) provide the aqueous pathways by which membrane bound structures can communicate and exchange necessary materials with their environment. This interaction is critical to a host of cellular and organellar functions. Thus, the control of channel permeability is of paramount importance to the regulation of the flux of matter across membranes and to the selective compartmentalization that is essential to biological function. Voltage-gating is one means by which channel permeability can be regulated. Simply defined, a voltage-gated channel is one for which the probability that it will be in a high-conducting state or a low-conducting state depends on the transmembrane potential. Voltage-gated channels are involved in a variety of vital activities, including, but certainly not limited to, respiration, nerve conduction, muscle contraction, development, and blood glucose regulation. However, despite the importance of voltage-gated channel regulation, the mechanism by which a channel protein detects and responds to an electric field across the membrane is not well understood.

VDAC is a voltage-gated, weakly anion-selective channel (2:1, Cl^- over K^+) located in the outer membranes of the mitochondria of all eukaryotic organisms examined thus far (protists: Schein, Colombini and Finkelstein, 1976; mammals:

Colombini, 1979, Linden, Gellerfors and Nelson, 1982; Roos, Benz and Brdiczka, 1982; plants: Zalman, Nikaido and Kagawa, 1980; Smack and Colombini, 1985). In the presence of a low potential (< +/-20 mV) the channels assume a high conducting "open" state but at higher potentials (> +/-20 mV) the channels are in a low conducting, "closed", state (Schein et al. 1976). The basis for this change of conductive properties is the change in the conformation of the channel due to the magnitude of the electric field. How the channel senses the electric field and how this results in conformational changes is unclear. However, a number of the properties of the VDAC channel make it an excellent protein in which to study the phenomenon of voltage-gated channel regulation: 1) its single channel conductance is large (4 nS in 1 M KCl) (Colombini, 1980a; 1986), 2) it has no major aqueous domains (Mannella and Colombini, 1984), 3) is easily isolated from a variety of organisms, and 4) can be readily reconstituted into planar phospholipid bilayers (Schein et al., 1976; Colombini, 1986). Knowledge of the voltage-gating mechanism of the VDAC channel has advanced rapidly.

The protein has two switching regions, one at positive and one at negative potentials and at least 2 closed channel states have been identified (Schein et al., 1976; Colombini, 1986). Evidence to date, largely from pH titration and succinic anhydride modification studies, indicates that most probably a group of at least 5 lysine epsilon amino residues is the portion of the protein that senses the electric field (Bowen, Tam and

Colombini, 1985; Doring and Colombini, 1985a; Adelsberger-Mangan and Colombini, 1987). In the closed state these gating charges are more accessible from the negative side of the channel (Doring and Colombini, 1985b). In addition, thus far, all treatments that alter the voltage sensing ability of the channel also alter the ability of the channel to select anions over cations (Doring and Colombini, 1985a; Adelsberger-Mangan and Colombini, 1987).

Molecules tend to assume their lowest energy state. If a molecule can assume multiple conformations, then the probability of the molecule existing in any given state depends on the energy difference between the states relative to thermal energy (Boltzmann distribution). Indeed, from the equilibrium probabilities one can calculate the energy differences between the conformational states. In the case of VDAC, the open state is the lowest-energy conformation in the absence of an applied membrane potential since it is the state in which the channels are found almost exclusively. The higher energy closed-channel conformations are attained with increasing probability as the magnitude of the electric field is increased.

Therefore, the electric field reduces the energy difference between the open and closed states until, at high fields, the low conducting conformation is the lowest energy form. The rate with which the channels assume one or the other state under given circumstances is a reflection of the time required by the channel to undergo the needed conformational reordering and that time depends on energy needed to accomplish these molecular changes.

Thus, analysis of the rates of channel opening and closing is a prime source of information on the mechanism of voltage-gating.

I have determined the rate constants of VDAC channel opening and closing. Analysis of the voltage dependence of the the rate constants provides new insights into the molecular mechanisms of VDAC's voltage-dependent behavior.

MATERIALS and METHODS

VDAC Preparation VDAC was obtained from the mitochondrial membranes of a wall-less mutant (slime) of Neurospora crassa (ATCC# 32360). Cells were maintained, cultured and harvested as described by Mannella (1982). Mitochondrial membranes were isolated by previously described methods (Mannella,1982) with slight modification. Briefly, mitochondria were isolated by differential centrifugation and hypoosmotically lysed to release soluble proteins. The total membrane preparation was concentrated by centrifugation. A crude fraction was prepared by resuspending the membranes in 1 mM KCl, 1 mM HEPES (pH 7.0). Occasionally an outer membrane fraction was prepared as described by Mannella (1982). There was no detectable difference in the behavior of VDAC derived from either preparation. All membranes fractions were stored in 15% (vol/vol) dimethylsulfoxide (DMSO) to a final concentration of approximately 3 mg protein/ml. Membrane preparations were stored at -70°C and aliquots were transferred to -20°C for short term storage prior to use.

General Methods The following methods were used throughout these studies. Exceptions to these, and details of particular experiments are presented, with the rationale, in the RESULTS. Experiments were conducted on planar phospholipid membranes prepared by the monolayer method of Montal and Mueller (1972) as described by Schein et al.(1976). Membranes were formed from soybean phosphlipids (Type-II-S, Sigma Chemical Co.), purified and stored as previously described (Kagawa and Racker, 1971). Aqueous 1M LiCl, 5 mM CaCl2 bathed both sides of the membrane.

All experiments were conducted under voltage-clamp conditions using a high-quality amplifier in the inverted mode (as described in Schein et al., 1976). A pair of calomel electrodes were used to interface with the aqueous phase. The trans side of the membrane was maintained at virtual ground by the electronics and therefore all potentials refer to the cis side. Current flow across the phospholipid membrane was recorded continuously on a Kipp and Zonen BD41 chart recorder and at appropriate times 2000 points were captured with a digital oscilloscope (Model 206, Nicolet Electronics. Inc.) and stored on a floppy disk.

The studies on the channel closing kinetics were conducted using an Analog Devices 52K operational amplifier with a 10^7 feedback resistor. No additional signal filtering was used. For these studies, the voltage was applied to the cis side by the use of manually-controlled switches. A potentiometer was used to select the desired voltage. Under these conditions an applied

voltage step from 0 to -60 mV was stable at -60 mV within 30 msec.

The opening process for VDAC channels is an extremely fast event. Therefore, modifications were made in the electronics in order to reduce electronic noise and increase instrument response time. An amplifier was used with a much faster time response: Analog Devices 50K. It was powered by a battery (+/-14 V) rather than a DC power supply. The current signal was low-pass filtered at 100 kHz before it was recorded. Voltage steps were applied to the cis side of the membrane by using the electronic switches of a Pulsar +7 pulse generator (Frederick Haer and Co.). All changes in the timing and magnitude of the applied voltage were pre-set and electronically executed. Under these conditions a step from 0 to -60 mV gave a stable -60 mV trans-membrane potential within 15 μsec.

Once a phospholipid membrane was formed it was evaluated to determine whether its properties were acceptable for further experimentation. The membrane had to have a low conductance (< 100 pS) and be stable in the presence of a high applied electrical potential. The stability of the membrane was assessed by pulsing to +/-10 and 40 mV for brief periods. A -40 mV potential was then applied across the membrane for several minutes. The trans-membrane potential was returned to -10 mV and an aliquot of the mitochondrial membrane preparation, solubilized in 1% Triton X100, (usually 10 μl) was added to the cis side while the solution was being stirred.

The spontaneous insertions of VDAC channels into the membrane were evident as step-wise current increases. Since the opening and closing of VDAC channels is a probabilistic phenomenon, a large number of channels were needed in the membrane in order to monitor the reproducible, average behavior of an ensemble. A minimum of 100 channels were needed in the membrane to obtain a smooth, analyzable record of the change in membrane current during channel opening and closing. However, it was necessary to control and, ultimately stop, channel insertion for several reasons. First, unbridled channel insertion leads to an exceedingly large membrane conductance which could lead to electrode polarization and reduce the resolution at which the current changes could be recorded. Secondly, the analysis employed in this work assumes that the change in membrane current observed is the result of the opening and closing of VDAC channels. Continued channel insertion could invalidate this approach. Therefore, channel insertion was controlled by adding an aliquot of a liposome solution (10% w/v asolectin in water) to the cis side of the membrane. Further additions were made (usually a total of 20 to 30 µl were needed) until the membrane conductance was stable for about 5 min.

DATA ANALYSIS

The rate constants of channel opening and closing were determined from the recorded current changes across multi-channel membranes when the trans-membrane potential was stepped to either an opening or closure-inducing voltage. It can be shown that the

rate constant (k) of opening or closing can be given by

$$k\ t = \ln \frac{(GI-GF)}{(Gt-GF)} \qquad (1)$$

where: GI, the initial membrane conductance, is the conductance of the membrane at the instant the potential change occurs. GF, the final membrane conductance, is the membrane conductance when steady state is achieved. Gt, the membrane conductance at any time t. Voltage steps were chosen so that the channels in the membrane would go from essentially all open (initially) to all closed (at steady state) or vice versa.

The experimental data was fit to this equation in order to obtain estimates of the rate constants. In theory, a plot of the conductance expression above as a function of time should yield a straight line whose slope is the rate constant. In practice, the data followed a straight line initially and then deviated. The slope of the initial straight line was taken as a measure of the rate constant of the process (see Fig. 12 insert).

The Channel Closing Rate Constant

The membrane was clamped at a potential at which most of the channels are open followed by a step change to a voltage at which most of the channels are closed. The event was captured on a digital oscilloscope. GI was determined from the 5 data point collected prior to the step change in potential. With the application of moderate potentials (-60 mV), GF could be obtained from the current level when steady state was achieved (5-7 min). However, GF was difficult to obtain when lower

potentials were applied because of the long time (>10 min) necessary to reach steady state. Extended exposure to high voltages resulted in membrane instability and thus GF was difficult to estimate. However, I have observed that the ratio GI-GF/GI is constant for a given membrane. Therefore, GF was determined with a voltage step to -60 mV and this ratio was determined and used to calculate GF from GI for the other voltage steps.

The fit to equation 1 was performed as follows. GF was electronically subtracted from GT, log transformed, and recorded as a function of time on an x-y plotter (Plotamatic 715, Allen Datagraph, INC.). The best fit line was drawn by eye and the rate constant calculated from the slope (see Fig. 12 insert).

The Channel Opening Rate Constant The membrane was clamped to a potential at which most of the channels are closed. After sufficient time was allowed for channel closure, the voltage was stepped to a potential at which most channels are open. The opening event was captured on a digital oscilloscope. GI was obtained from data points taken just before the step change to an opening voltage. GF was obtained from the conductance achieved at steady state.

The time course of channel opening overlapped in time with the current resulting from the capacitive charging of the membrane. Therefore, Gt as a function of time was obtained by subtracting the capacitive current across the unmodified

(channel-free) membrane (scaled for any difference in the size of the voltage step) from the recorded current flow across the multi-channel membrane. Since each record consisted of up to 1800 points, this subtraction was computer assisted!! A Basic program was designed to: 1) transfer the data from the files stored by the digital oscilloscope and convert them to current values, 2) align the capacitive and the multi-channel records in time, 3) if desired, perform some averaging to reduce the noise by reducing the time response, and 4) store the data in a CSV file format so that they could be imported into a spread-sheet program (Supercalc 4). A spread-sheet program was designed to conduct all further manipulations.

Steady State Voltage Dependence Parameters The steady state voltage-dependent properties of the channels were analyzed by the method introduced by Ehrenstein (1970) for the study of EIM channels and employed for VDAC channels by Schein et al.(1976)(see THEORY for details). Briefly, the probability of channels being in the open or closed state at a given applied voltage across a multi-channel membrane is given by:

$$\ln(G-GF/GI-G) = (-nFV+nFV_o)/RT \qquad (2)$$

The measurements of multi-channel membrane current recorded as a function of applied voltage were converted to conductance values as a function of voltage using a Hewlett-Packard digitizer and HP-85 computer. A measure of the steepness of the voltage dependence, n, and the voltage at which half the channels are open, V_o, were then obtained from the slope and y-axis intercept

of the plot of ln (G-GF/GI-G) vs. voltage.

THEORY

The two-state model VDAC is known (Schein et al., 1976) to occupy a high-conductive "open" state at low membrane potentials and low-conductive "closed" states at high potentials. It is known (Colombini, 1986) that the closed states occupied at positive potentials are different from those occupied at negative potentials. Thus two gating processes occur, one at positive potentials and one at negative potentials. The behavior of the gating processes is almost identical and I have chosen to analyze the one that occurs at negative potentials.

From single channel conductance measurements it is clear that there are a variety of different closed conductance states at both positive and negative potentials. The process will be modeled by a single closed state for the following reasons: 1) to include the variety of closed states observed into the model would introduce an unacceptable number of free parameters; 2) the use of multi-channel-membrane experiments does not allow the investigator to distinguish between transitions from the open state to each closed state or among closed states; and 3) the properties of VDAC preclude quantitative kinetic analysis with single channels. The following two-state model was used:

$$\text{open state} \underset{k_o}{\overset{k_c}{\rightleftharpoons}} \text{closed state}$$

At equilibrium the number of channels in either the open or closed states reflects the energy difference between the two

states. Since the equilibrium constant, K, is given by the ratio of the closing rate constant, k_c, to the opening rate constant, k_o, the steady state energy difference, ΔE, between the open and closed state can be given by:

$$\Delta E = -RT\ln K \quad (3)$$
$$= RT\ln k_o - RT\ln k_c \quad (4)$$

where R and T are the gas constant and temperature, in degrees Kelvin, respectively. In the case of VDAC, the increased transmembrane potential shifts the equilibrium constant in favor of the closed-channel state. Thus, the voltage dependence of the VDAC channels observed in the steady state must be reflected in either or both of the rate constants of channel opening or closing.

I have described and quantified the voltage dependence of the channels using the methods first introduced by Ehrenstein (1970) for the analysis of the EIM channels and later employed by Schein et al. (1976). The Boltzmann distribution describes the probability of the channels being in either the open or closed state as a function of the energy difference between the states:

$$\frac{N_o}{N_c} = \frac{\text{number open}}{\text{number closed}} = e^{\Delta E/RT} \quad (5)$$

In the case of a voltage-dependent channel the energy difference between the states, ΔE, has a voltage-dependent component as well as a voltage-independent component attributable to the inherent difference between the two conformations. Further analysis of these components assumes that the conformational change from open to closed is accompanied by the

movement of charged groups, on the channel, through all or part of the membrane potential. Consequently, the energy difference between the open and closed states of VDAC can be given by the algebraic sum of the energy involved in the movement of charge (nF) through a potential difference (V) and the energy difference between the states in the absence of a voltage (nFV_o) such that:

$$\Delta E = -nFV + nFV_o \qquad (6)$$

where F, and V, are Faraday's constant and the applied voltage respectively. V_o is the voltage at which half the channels are open (i.e. at which ΔE is zero) and n is the minimum number of charges that would have to move through the entire electric field in order to account for the observed voltage-dependence and can be viewed as a measure of the steepness of the voltage dependence. If the applied voltage, V, equals V_o then the energy level of the two states is equal and neither state is energetically favored. At applied voltages above V_o, the probability of the channels being in the closed state increases and similarly at applied voltages below V_o, the open state is favored. In the absence of an applied field, V=0, the energy difference between the states is the inherent conformational energy difference and the energy required to compensate for this is nFV_o. Thus, nFV can be used to describe and quantify the voltage dependent component of the channels and nFV_o can be used in a similar manner for the voltage independent component.

This approach seems valid for the VDAC channel since there is strong evidence that charged groups (most likely lysine residues)

are responsible for sensing the potential and allowing VDAC to respond to changes in the potential. Voltage-dependence can be reduced by chemical modifiers as well as by titration. Succinic anhydride, an amino group modifier, essentially eliminates VDAC's voltage dependence (Doring and Colombini, 1985a, Adelsberger-Mangan and Colombini, 1987). Increasing the pH of the medium bathing the channels from 6.2 to 10.7 markedly reduced the voltage dependence of the channels as well (Bowen et al.,1985).

The voltage dependence of the overall process can be divided into the voltage dependence of the individual reactions:

$$nFV = n_cFV + n_oFV \qquad (7)$$

Therefore, using E_b as a measure of the energy barrier to opening in the absence of a field and combining equations 4, 6, and 7, yields expression of the relationship of the rate constants of channel opening and closing to the voltage-dependence of the channels:

$$\Delta E = E_c - E_o = -n_cFV + E_b + nFV_o - (n_oFV + E_b) = RT\ln k_o - RT\ln k_c \qquad (8)$$

And therefore, for the channel closing process

$$E_c = ncFV + E_b + nFV_o = -RT\ln k_c + C \qquad (9)$$

$$\ln k_c = n_cFV/RT - (E_b + nFV_o + C)/RT \qquad (10)$$

Thus, an estimate of n_c, the steepness of voltage dependence of the closing rate constant, can be obtained from the slope of a plot of the log transformed rate constants as a function of the applied voltage. A similar approach could be used to approximate the voltage dependent component of the channel opening process.

RATE CONSTANTS FROM MULTI-CHANNEL MEMBRANE CONDUCTANCES

For VDAC channels, a change in conductance is the obvious indicator of the change of a channel from one state to another. In the planar bilayer system used in these studies virtually all the conductance across a multi-channel membrane is due to VDAC channels. Therefore, I have used the change in conductance across multi-channel membranes to approximate the rate constants of VDAC opening and closing.

After integrating the rate equation of the two state system, the rate constant of closure, k_c, is given by:

$$k_c\ t = \ln\ (NT/NT-N_c) \qquad (11)$$

Where: NT = Total number of channels

N_c = Number of closed channels

N_o = Number of open channels

Since the total number of channels, NT, is the sum of the number open, N_o, and closed, N_c, the rate expression reduces to

$$k_c\ t = \ln((N_c+N_o)/N_o) = \ln\ [1+(N_c/N_o)] \qquad (12)$$

Since changes in membrane conductance are monitored, the following relationships can be used to relate the number of channels in the open or closed state to the membrane conductance.

Conductance at time t = $Gt = N_o g_o + N_c g_c$

Initial Conductance = $GI = NTg_o$

Final conductance = $GF = NTg_c$

Where g is the channel conductance in the open (g_o) or closed (g_c) state. From these relationships it can be shown that:

$$N_c/N_o = (GI-Gt)/(Gt-GF)$$

Therefore, the rate constant can be obtained from the conductance changes across a multi-channel membrane as follows:

$$k_c\ t = \ln\left(1 + \frac{(GI-Gt)}{(Gt-GF)}\right) \qquad (13)$$

$$= \ln((GI-GF)/(Gt-GF)) \qquad (14)$$

In theory, a plot of the conductance changes in equation 14 as a function of time is a straight line through the origin. The rate constant of channel closure is the slope of the resulting line. The same argument can be made for determination of the channel opening rate constant.

The steady state voltage dependence parameters, n and V_o can be obtained from multi-channel membrane conductances in a similar manner. Using the conductance relationships presented above and equations 5 and 6, the voltage dependence parameters can be related to the steady state conductance at any applied voltage (G) as follows:

$$\ln\ (N_o/N_c) = \ln\ (G-GF/GI-G) = (nFVo - nFV)/RT \qquad (15)$$

Analysis of the plots of the log transformed steady state conductance entity in the above expression as a function of the applied voltage yields n and V_o.

RATE CONSTANT ANALYSIS

Many chemical reactions are described in terms of Eyring rate theory. The reactants and products are viewed as resting in energy wells on either side of one or more energy barriers. In the formation of product, the reactants move over the barrier(s) from one well to the other. The height of the barrier, the

"activation energy", is the energy which must be acquired in order to form the "activated complex", a high-energy form which leads to product formation. The rate at of product formation depends on the rate of activated complex formation which, in-turn, is proportional to the required activation energy.

For the two-state model of VDAC voltage-dependence, an Eyring model with two wells and a single barrier, is appropriate. Voltage dependence is then viewed as a voltage-dependent change in the energy level of the states of the channel: the open, the closed and the activation complex. Since an increase in the membrane potential induces VDAC closure, the potential must reduce the energy level of the closed state relative to the open state. The energy level of the activation complex is probably affected in an intermediate manner since it is a state intermediate between the open and closed states. However, this is not necessarily the case. Irrespective of the energy level of the activation complex, the algebraic sum of the voltage dependence of the energy differences between open state and the activation complex and the closed state and the activation complex, must be equal to the voltage dependence of the energy difference between the open and closed states.

Eyring and others (Eyring et al.,1949; Glasstone et al., 1949) formulated the relationship between the rate constant of a chemical reaction and the potential energy barriers that must be overcome in order for a given reactant to become the desired product (it relates the rate constant to the energy of

activation). Consider the closure process such that E_c is the potential energy barrier to closure. The relationship of the energy barrier to the rate constant of channel closure can be given by:

$$k_c = \frac{TK}{h} y\, e^{-E_c/RT}$$

T, K, h, and R are absolute temperature, the Boltzmann, Plank, and gas constants respectively. The entity TK/h is the universal frequency factor and has the value of 5.8×10^{-12} sec^{-1}.] This factor specifically describes the frequency with which the activated complex decomposes to product. It can be replaced by a single term, Q. y is the transmission coefficient that reflects the probability that the activated complex will be transformed to the desired product. Its values can range between 0 and 1 for any given reaction. However, it is not usually possible to determine its value and, therefore, is assumed to be very near unity. In the absence of any compelling reasons to do otherwise I followed this convention and assumed y was 1. Therefore, for a two-state model of the VDAC channel, the energy barrier that must be overcome in the transition from the open state to the closed state can be given by:

$$E_c = -RT\ln k_c + RT\ln Q \qquad (17)$$

Therefore, having determined the rate constant for channel closure at a given applied voltage, the magnitude of the potential energy barrier between the open and closed states at that voltage could be calculated. Certainly these estimates will

reflect the numerous simplifying assumptions made in the analysis of the rate constants .

RESULTS

VDAC channels are open at low transmembrane potentials and closed at high potentials. The transmembrane voltage, therefore, shifts the equilibrium constant between the two states of the channel in favor of the closed state. This voltage dependence of the equilibrium constant must be reflected in a voltage dependence of the opening and/or closing rate constants (see THEORY). The rate constants of channel opening and closing were determined by applying step changes in the potential across multi-channel membranes and following the rate of either channel opening or closing. Channel closing rates were measured following a step increase in the transmembrane voltage and opening rates following a step decrease in voltage.

Closing kinetics Planar phospholipid membranes were generated and sufficient numbers of VDAC channels were inserted (>100) in order to obtain fairly smooth kinetic curves (to avoid the stochastic behavior observed with few channels in the membrane). Membrane current was monitored at -10 mV. The potential across the membrane was then dropped to zero for 6 min. The transmembrane potential was then stepped to a closure-inducing voltage (-30,-40,-50,-60,-70, or -80 mV) for 3 min. The current through the membrane was monitored on a chart recorder and the initial current change (100 sec for -30 and -40 mV; 20 sec for -50 and - 60 mV and 10 sec for -70 and -80 mV) was recorded and

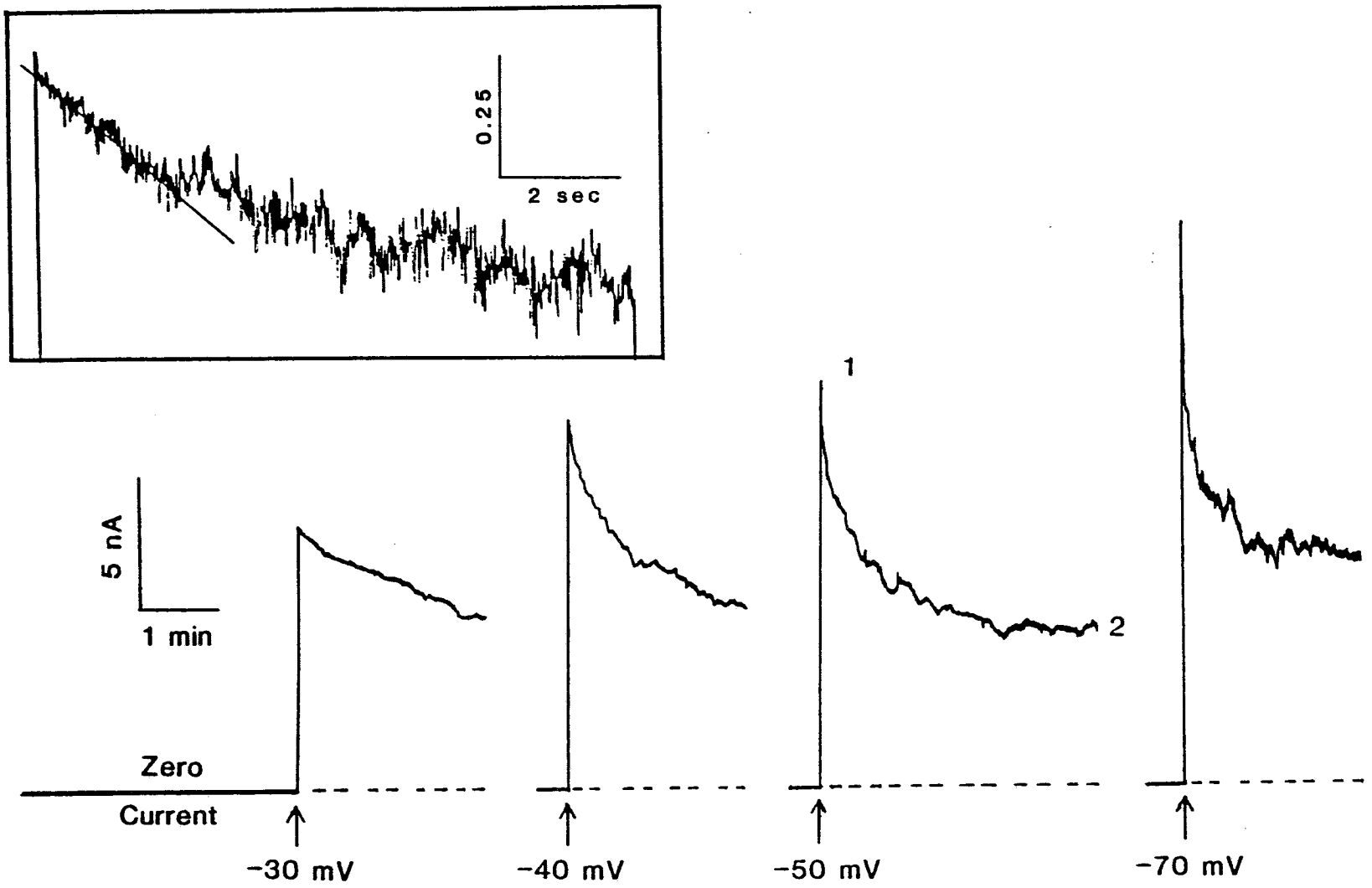
stored on floppy discs via a digital oscilloscope. The rate constants of closure were determined from the digital records of the decrease in membrane current . After 3 min at the closure voltage, the transmembrane potential was returned to zero for 6 min and the process repeated at another closure voltage. Most often, within 30-40 sec before and after the closure step the potential was briefly pulsed to -10 mV. The -10 mV current level was used to detect changes in membrane conductance (usually due to channel insertion) and GI was calculated by simple linear adjustment of this conductance for the increased driving force. A closure-inducing voltage was applied and the current flowing through the membrane was recorded as a function of time.

Examples of the results obtained with one such membrane are shown in Figure 12. In the absence of an electric field (0 mV) the channels were in the high-conducting open state. No current flowed due to the absence of a driving force. When the voltage was stepped to a higher, closure-inducing potential, the membrane current instantaneously increased to a level reflecting the increased driving force across the, still open, channels. GI was calculated from this initial current level. The membrane current then decreased as the channels began to close and continued to fall until essentially all the channels were closed. The residual current level reflects the current through the closed channels at the applied voltage (Fig. 12, -50 mV) and was used to determine final membrane conductance (GF). Typically GF was 40-50% of GI.

Figure. 12 Increasing the applied voltage increases the rate of channel closure. Membrane current flow across the same membrane containing many VDAC channels is depicted at negative closure-inducing potentials (-30, -40, -50, -70 mV). Salient features are illustrated in the -50 mV record. Initially, there was no applied voltage across the membrane and thus, no current. At the arrow the voltage was stepped to -50 mV, and the current flow recorded. The initial current level is indicated at 1. The instantaneous open channel conductance at the closure voltage, GI, could be calculated from this current level. The current decreases with time as the channels close to a baseline level indicated at 2. This is the membrane current when essentially all the channels are closed and the corresponding conductance, GF, was calculated from this current level. Insert: Log transformed data of a typical experiment fit to equation (1) in the form:

$$\ln (Gt-GF) = \ln (GI-GF) - kct$$

The best fit line through the initial conductance change was drawn by eye and the rate constant calculated from that slope. The initial log transformation was to the base 10, thus, the ordinate is in base 10 units. Values, therefore, were multiplied by 2.3 to obtain the natural log values. Room temperature, 1 M LiCl and 5 mM $CaCl_2$.


0.25
2 sec
1
2
5 nA
1 min
Zero
Current
-30 mV
-40 mV
-50 mV
-70 mV

The rate at which the current across a multi-channel membrane dropped to GF increased as the magnitude of the applied voltage was increased (Fig. 12). Thus, the closing rate constant is voltage dependent. In order to quantitate the rate constant I needed to consider two issues: 1) the multi-exponential nature of the phenomenon, 2) the variability of the closure rates. As to the first issue, I chose to examine the first rate constant. The second was dealt with by trying to minimize the number of variables.

One possible source of the variation appeared to be the amount of time between the closure pulses. Therefore, I tested the possibility that the amount of time the channels were in the open state, before the step increase in voltage, might alter the rate of closure. Perhaps the channels could occupy multiple open states and the open state first occupied by a newly-opened channel may not be the most-favored state. A multi-channel membrane was prepared as previously described and a -60 mV potential was applied for 2 min. The voltage was then dropped to 0 mV for 0.5 min. The potential was then stepped to -60 mV for 2 min, the resulting current change recorded and the rate constant determined. This sequence was repeated for 1, 2, 4, 6, and 8 min intervals at 0 mV. Control rate constant values were obtained from current changes of membranes which had never been exposed to a closure voltage or from those maintained at 0 mV potential for 30 min.

Figure 13 shows the effect of time in the open state on the rate constant of VDAC closure. The rate constant was markedly reduced following only o.5 to 1 min periods in the open state. However, the rate constants essentially returned to control values following 6 min in the open state and further time in the absence of a potential did not appear to effect the estimates. As a result of these findings, closure rate constants were determined from channel closures preceded by at least a 6 min interval in the open state.

Another potential source of variability of the rate constants is the number of channels present in the membrane. Cooperativity between channels could alter the rate constants. Any cooperativity would depend on the number of channels in the membrane and, therefore, on the total membrane conductance. The possibility that variation in the number of channels in the membrane might result in variation in the measured rate constants was examined by plotting the rate constants obtained from membranes with a range of conductances. Figure 14 clearly indicates that the VDAC closure rate constants are independent of the conductance of the membrane from which they have been determined.

The closure rate constants, as a function of applied voltage, were determined in several experiments and the results plotted in Figure 15. Despite some residual variability, the results were very reproducible. As expected, the closure rate

Figure 13. Time in the open state effects the rate of VDAC channel closure. The rate constants of channel closure at -60 mV were determined following increasing periods of time in the absence of a potential (open state). Control rates were determined on membranes that had never been closed or had remained in the open state for 30 min. Bars are the standard deviations of 4 experiments, each conducted entirely on one membrane. Conditions as in Figure 12.

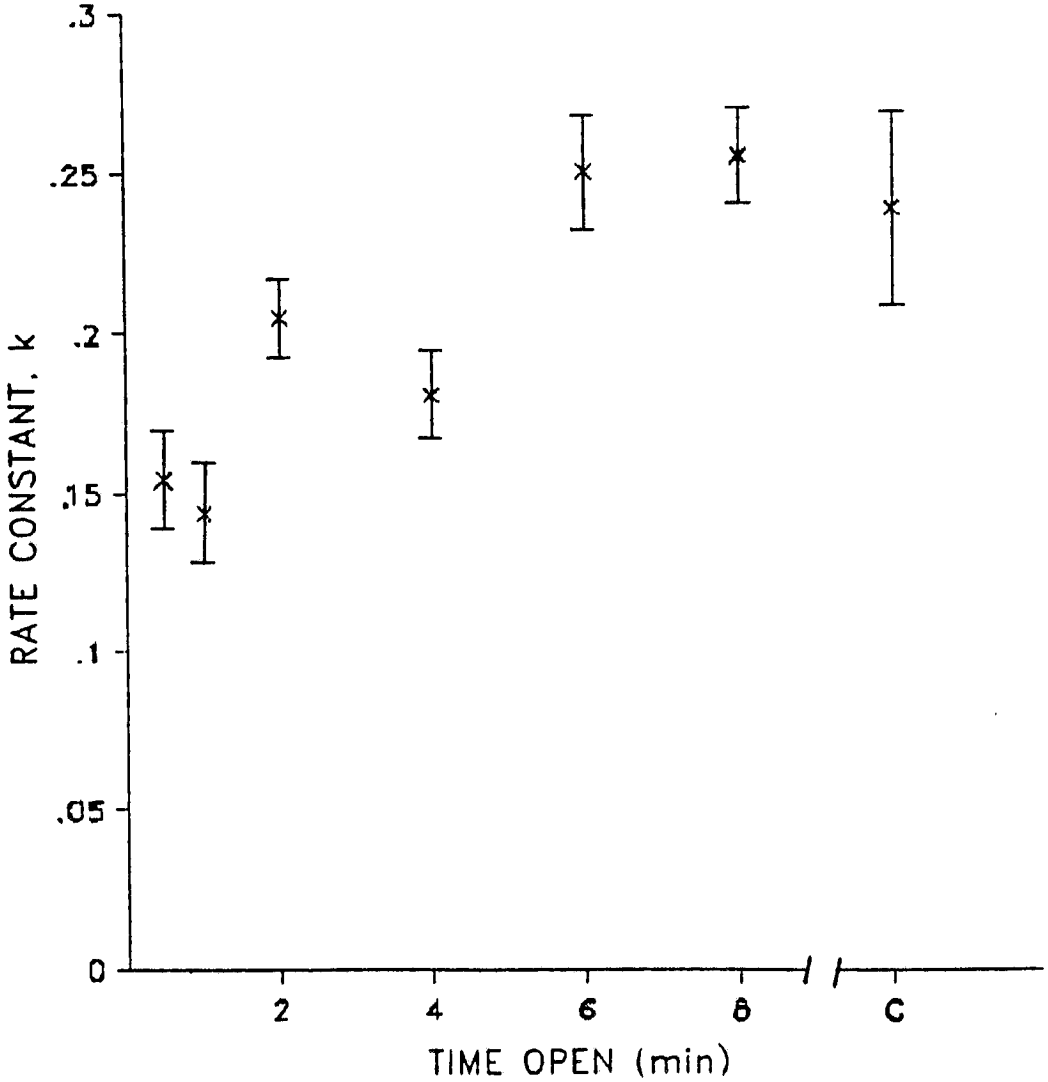

.3
.25
.2
.15
.1
.05
0
RATE CONSTANT, k
2
4
6
8
C
TIME OPEN (min)

Figure 14. The rate of channel closure is independent of the number of channels in the membrane. The log transformed rate constants from 5 experiments are plotted as a function of the membrane conductance. -30 mV (□); -40 mV (×); -50 mV (▽); -60 mV (⧻); -70 mV (◇).

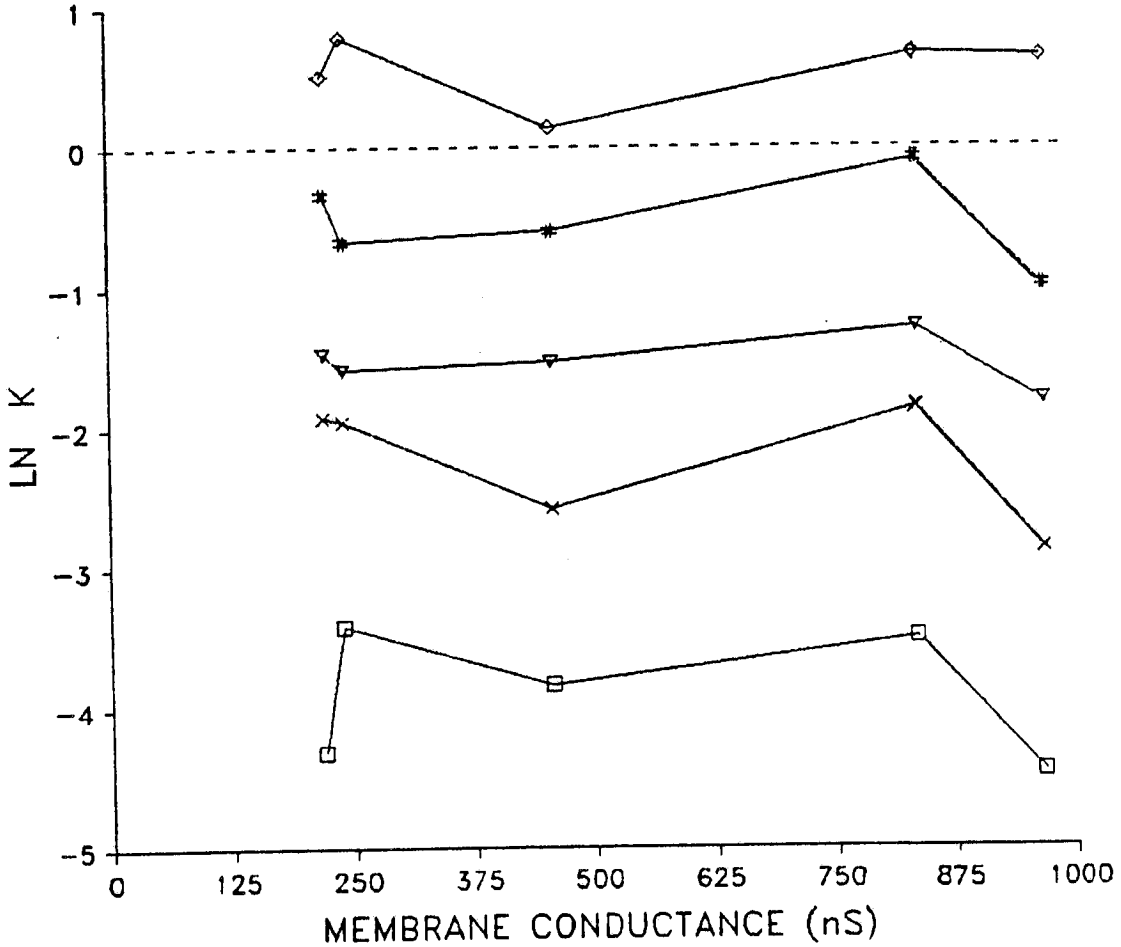
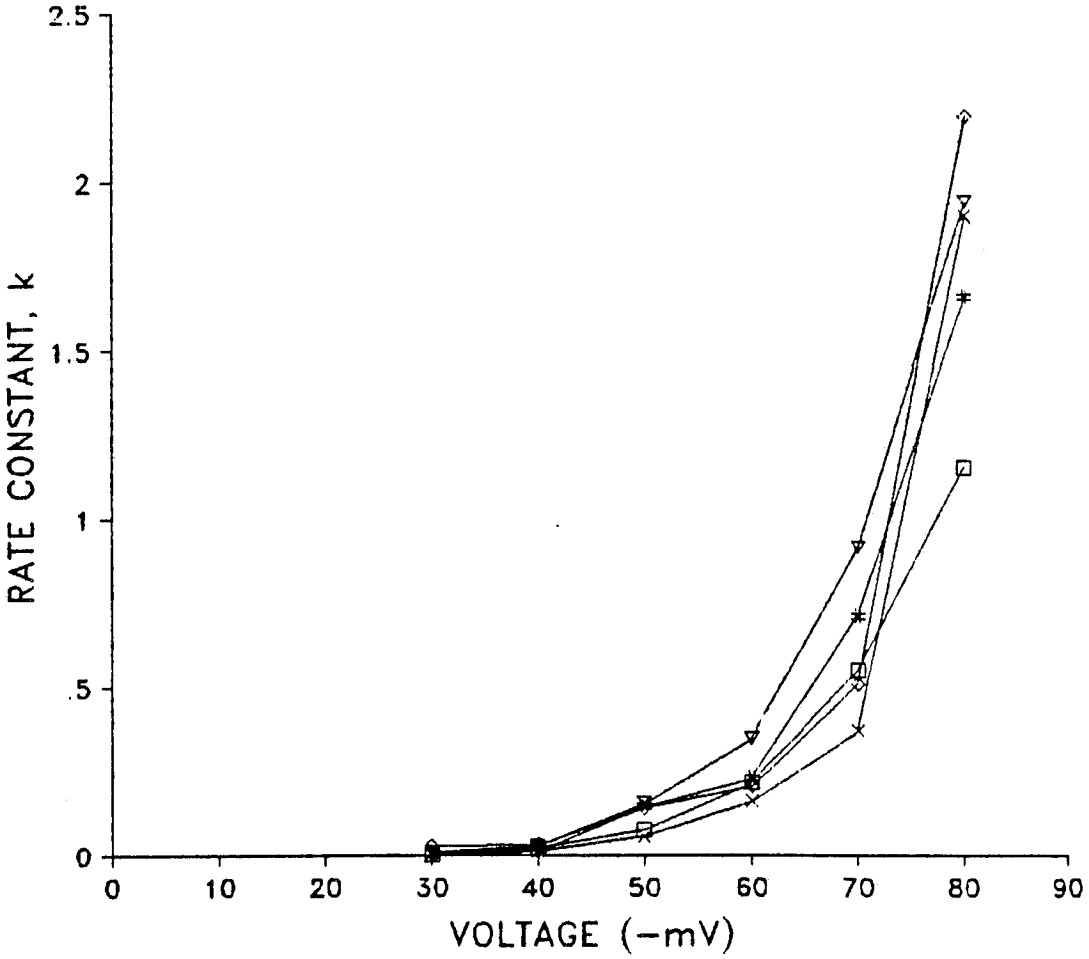

1
0
−1
−2
−3
−4
−5
LN K
0
125
250
375
500
625
750
875
1000
MEMBRANE CONDUCTANCE (nS)

Figure 15. The rate constants of VDAC channel closure increase exponentially with the magnitude of the transmembrane potential. The rate constants of channel closure were determined as described in the text from multi-channel membranes. The results obtained from 5 experiments, each conducted on a single membrane, are depicted. Conditions as in Figure 12.

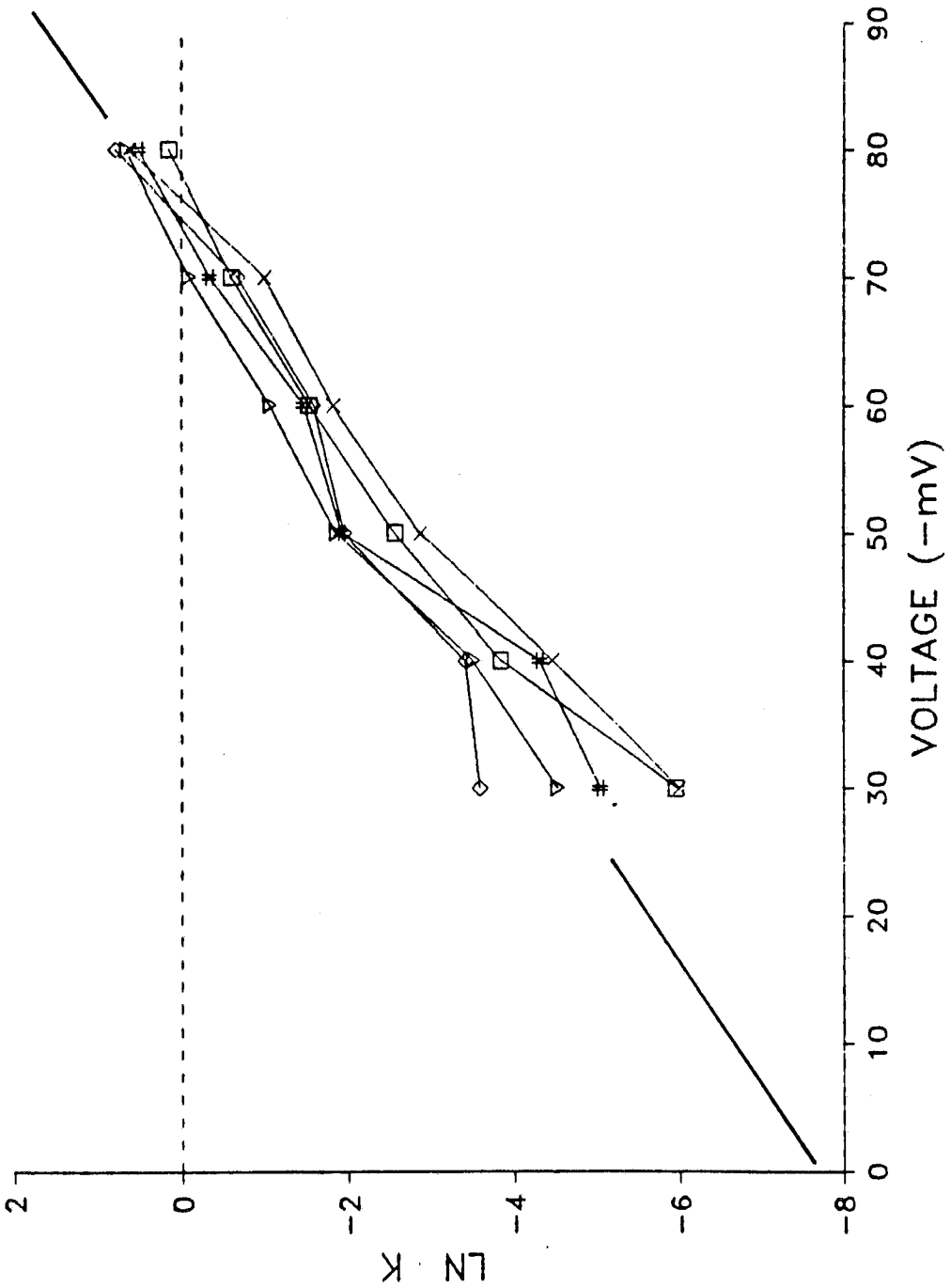

RATE CONSTANT, k
2.5
2
1.5
1
.5
0
0
10
20
30
40
50
60
70
80
90
VOLTAGE (−mV)

constants increased exponentially with increased applied voltage (Fig. 15). The mean rate constant of closure increased from 0.01/sec (+/-.002 S.E.) at -30 mV to 1.77/sec (+/-.078 S.E.) at -80 mV. Figure 16 shows that the log transformed rate constants from 5 experiments plotted as a function of applied voltage give a family of lines with a best fit line correlation coefficient of 0.99.

Opening kinetics It has long been observed that VDAC channels open much more rapidly than they close (Schein et al., 1976). I determined the rate constants from changes in the current following an essentially instantaneous voltage change across the membrane. Changing the potential across a membrane results in a very rapid capacitive current. Preliminary experiments indicated that that the channel opening occurred within the same time frame as the membrane capacitive current. Therefore, determination of the rate constants required the quantification of the membrane capacitance for each experiment.

A stable asolectin membrane was prepared as described but, prior to the insertion of VDAC channels, records of the capacitive current across the membrane at different applied voltages were obtained. The potential across the membrane was stepped to a closure-inducing voltage (usually -50 or -60 mV) for at least 20 sec and then it was dropped to a positive opening-inducing voltage (usually +10 mV). The resulting current flow was digitally recorded as described above. This protocol was repeated with a negative potential of the same magnitude. VDAC

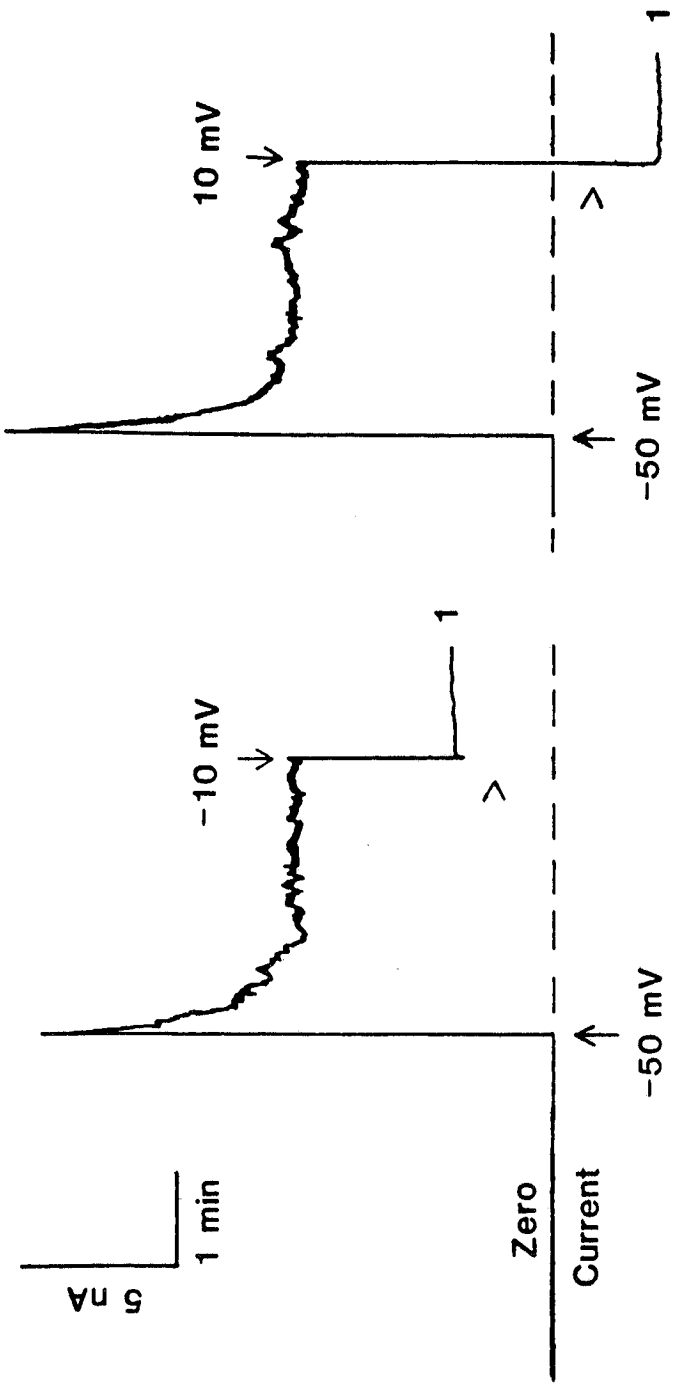
Figure 16. Linearized rate constants of VDAC channel closure. The rate constants shown in Figure 15 were log transformed and plotted as a function of the applied potential. The best fit line through the data is shown. From the slope of the line, the steepness of the voltage dependence of channel closure, n, equals 2.6 (see Text). (Y= 104.6X - 7.75; r =0.997) All conditions as in Figure 12.


LN K
2
0
-2
-4
-6
-8
0
10
20
30
40
50
60
70
80
90
VOLTAGE (-mV)

channels were then inserted and the membrane conductance stabilized with liposomes. The multi-channel membrane was maintained at zero potential for at least 5 min and the potential was stepped to a closure-inducing voltage (-50 or -60 mV). After 2 to 3 min the transmembrane potential was stepped to either a positive or negative opening voltage (+ or -5, and 10 mV, and -15 mV). The resulting current changes were digitally recorded. These recorded changes in the membrane current were a composite of the current change due to membrane capacitance and the current change resulting from VDAC channel opening in the presence of the reduced electric field.

Figure 17 shows the change in current flow across the membrane during such an experiment. Typical channel closure behavior was evident. Membrane current instantaneously increased before the onset of channel closure. The current decreased to a baseline level at which all the channels were essentially closed. At the arrow the voltage was dropped to open the channels. The current instantaneously decreased to an initial level which was the current through the, still closed, channels at the reduced driving force. From that current level GI was determined. The initial current change due to channel opening was so rapid that it exceeded the response time of the recorder. Arrow heads indicate the actual levels. The current then increased toward a level at which all the channels were open, and GF was determined from that current level.

Figure 17. Current records of VDAC channel opening. Two records of multi-channel membrane current changes during channel opening and closing are shown. Initially, there was no applied field for at least 5 min. At the arrow the potential was stepped to -50 mV to close the channels. When essentially all the channels were closed, the potential was stepped to an opening-inducing voltage. The rate of channel opening exceeds the limitations of the recorder and arrow heads indicate the instantaneous closed channel current at the new opening-inducing voltage from which GI was calculated. A stable current level was reached when essentially all the channels had opened (1) and GF was determined from this current.


5 nA
1 min
Zero
Current
-50 mV
-10 mV
1
-50 mV
10 mV
1

The overlap of the capacitive current with the changes in ionic current associated with channel opening is clearly illustrated in Figure 18A. A record of the membrane capacitive current was obtained on the membrane before channel insertion. The voltage was raised to a closure-inducing potential for 20 sec and then dropped to an opening-inducing potential. The current change which occurred when the voltage was dropped was digitally recorded. Channels were then inserted into the bilayer and membrane conductance allowed to stabilize. The channels were closed at -60 mV for 3 min an the trans-membrane potential was then dropped to -10 mV. The current immediately before and following the voltage drop was digitally recorded. Comparison of the two records shows the overlap between the changes in ionic and capacitive currents. Since the capacitive current resulted from the change in voltage across the membrane, the observed current change across the multi-channel membrane was the sum of the current change due to channel opening and that due to capacitance charging.

A protocol was developed to subtract the capacitive current from the total current changes recorded during channel opening. The remaining current, that due to channel opening, could then be used to determine the rate constant. The capacitive current across the unmodified membrane was recorded for each membrane.

Figure 19 illustrates that this subtraction can be done successfully. The capacitive current across an unmodified membrane was recorded when the voltage was dropped from -50 to -

Figure 18. The current across a multi-channel membrane during VDAC opening overlaps with the membrane capacitance current. Panel A: The capacitance current across an unmodified membrane resulting from a voltage drop from -50 mV to -10 mV was recorded (□). After channels were inserted, the current across the membrane during channel opening at -10 mV from a -50 mV closure potential (✕) was recorded. Panel B: The multi-channel membrane current after subtraction of the capacitance current from the total multi-channel membrane current shown in Panel A. The resulting membrane current changes are attributable to the channel opening. The fluctuations are within the typical noise level of approximately +/-0.3 nS. Each point is the average of 5 current values, each 10 μS apart. Insert: Log transformed conductance changes (see DATA ANALYSIS) derived from the experiment illustrated in Panels A and B plotted as a function of time. The membrane conductance, Gt, at a given time was determined from the current data in Panel B and used in equation 1 to determine the rate constant. The best fit line through the data is shown. The rate constant was the slope of this line. Other conditions as in Figure 12.

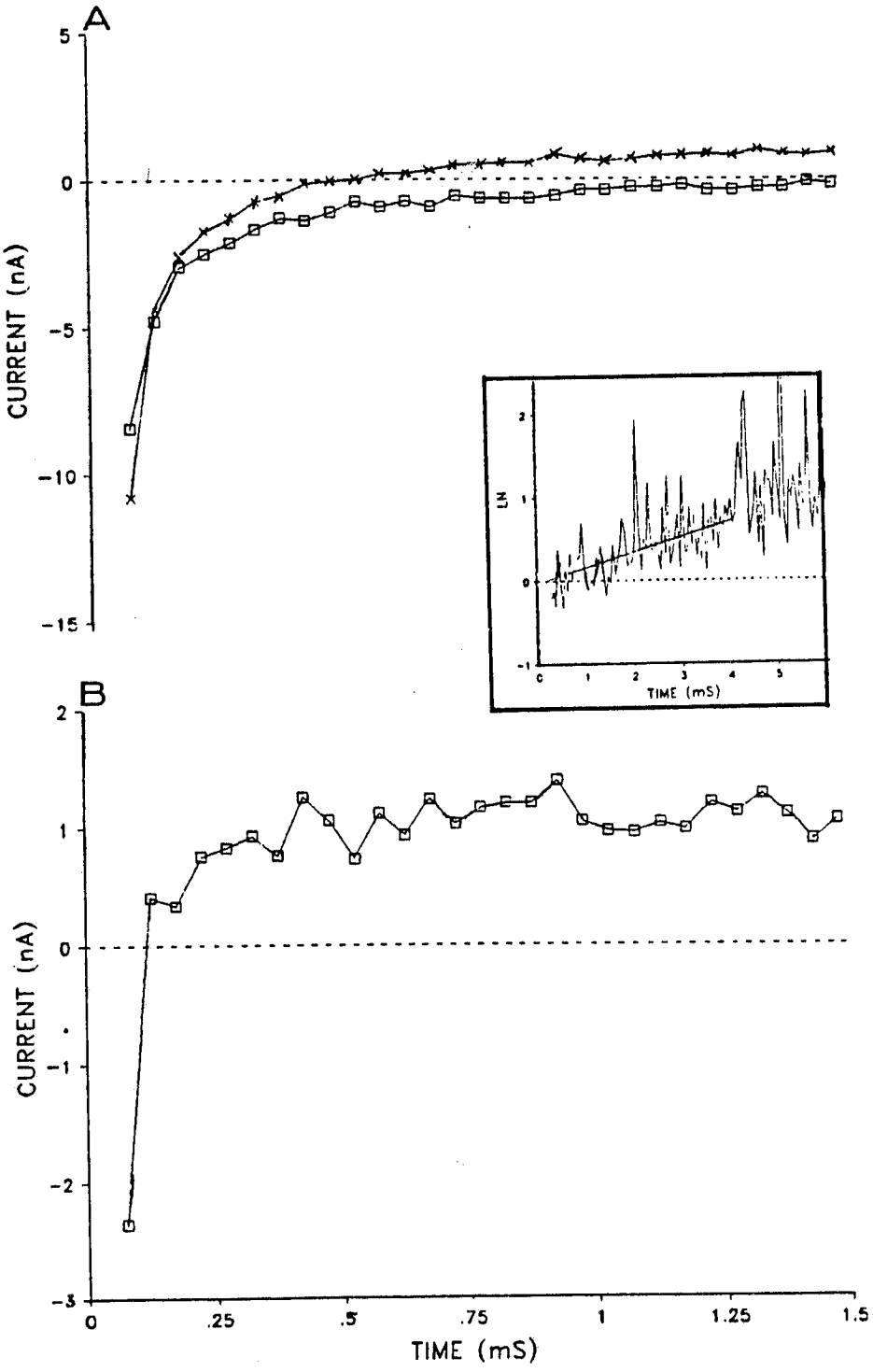
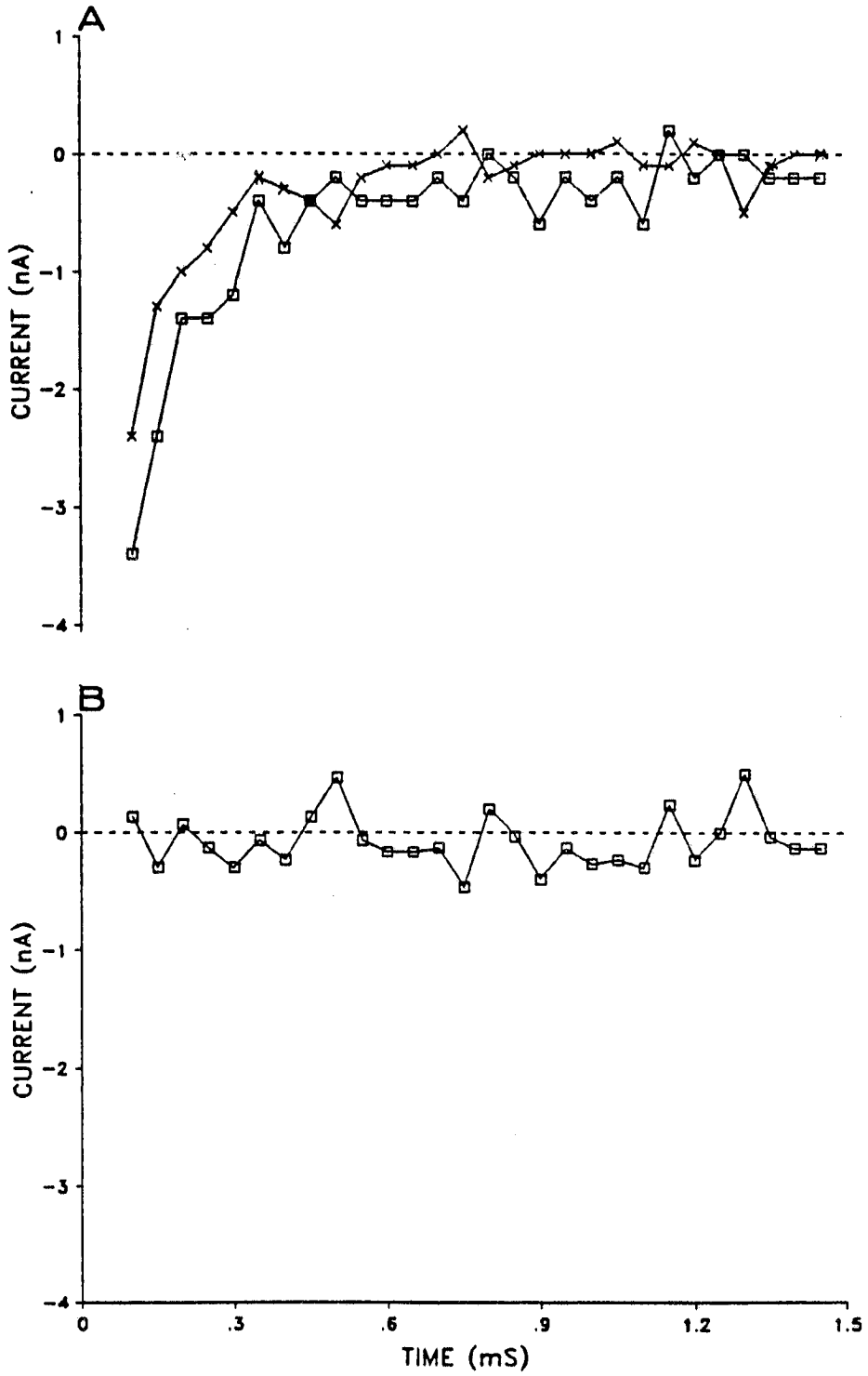

A
5
0
-5
-10
-15
CURRENT (nA)
LN
2
1
0
-1
0
1
2
3
4
5
TIME (mS)
B
2
1
0
-1
-2
-3
CURRENT (nA)
0
.25
.5
.75
1
1.25
1.5
TIME (mS)

Figure 19. Subtraction of membrane capacitance current from a second current record. Panel A: Capacitance current records obtained when the voltage was dropped from -50 mV to -10 mV (×) and from -50 mV to +10 mV (□). The +10 mV capacitance current values were adjusted for the difference in driving force and the -10 mV current values were subtracted. The resulting current is shown in Panel B. Conditions as in Figure 12.


A
B
CURRENT (nA)
CURRENT (nA)
1
0
-1
-2
-3
-4
0
.3
.6
.9
1.2
1.5
TIME (mS)

10 mV and from -50 to +10 mV (Fig. 19A). The current values recorded as a result of the drop to +10 mV were adjusted for the 20 mV difference in the magnitude of the voltage change between the two capacitive records by simple linear scaling. When the capacitance current curves were subtracted there was essentially no residual current (Fig. 19B). The current fluctuations are due to noise in the records and the fluctuations are about the zero current axis.

The results of this approach when applied to the analysis of a channel opening event are illustrated in Figure 18 (A,B). Panel A shows the total recorded current change across a multi-channel membrane with a -10 mV applied potential and the capacitive current across that same membrane in the absence of the channels. The difference, shown in panel B, is the current change from which the rate constants were calculated. The log transformed data is presented in the insert. The subtraction of the recorded capacitance current rarely eliminated all the displacement current and the source of the residual current was unknown. However, the initial membrane conductance due to VDAC channels was accurately known because I obtained GI from the digital records of steady state current values just prior to a voltage step. Therefore, regardless of the possible source of the residual current, I used the zero intercept to fit the best line to the transformed data.

The rate constants of channel opening were determined at both positive and negative potentials after the channels were

closed with a high negative potential (Fig. 20). Although there is wide variation in rate constants determined in different experiments, within a single experiment, at the level of resolution presently available, there is no marked voltage dependence of the rate constants between opening at -15 and -5 mV or between +5 and +10 mV. The opening rate constants range from 120/sec to 230/sec at -5 mV. But, opening the channels at a positive voltage increased the rate constants by an order of magnitude (Fig. 21)!. When opened at +5 mV, the rate constants increased dramatically to over 3.5 x 10^3 /sec. Sharp discontinuities between the opening and closing rate constants and channel opening at positive and negative potentials are evident in Figure 22. There are no obvious extrapolations which will result in equal opening and closing rates at V_o as might be expected in a two-state model.

The steady state voltage dependence parameters were determined by methods described elsewhere (c.f.Bowen et al., 1985). The voltage dependence of the steady state (essentially the equilibrium constant) was determined with multi-channel membranes bathed in 1 M $LiCl_2$ and 5 mM $CaCl_2$. A triangular voltage wave, +/-64 mV, was applied, the resulting current recorded and analyzed as described earlier. The steepness of the steady state voltage dependence was 3.2 (i.e. 3.2 would be the minimum number of charges need to account for the observed voltage dependence). The slope of the line of the log transformed closing rate constants as a function of voltage

Figure 20. Rate constants of VDAC channel opening as a function of applied voltage. Rate constants were obtained from multi-channel membranes as described in the text. Results of 3 experiments, some with replication, are presented: (□,≠), (△,X), (▽,╬).

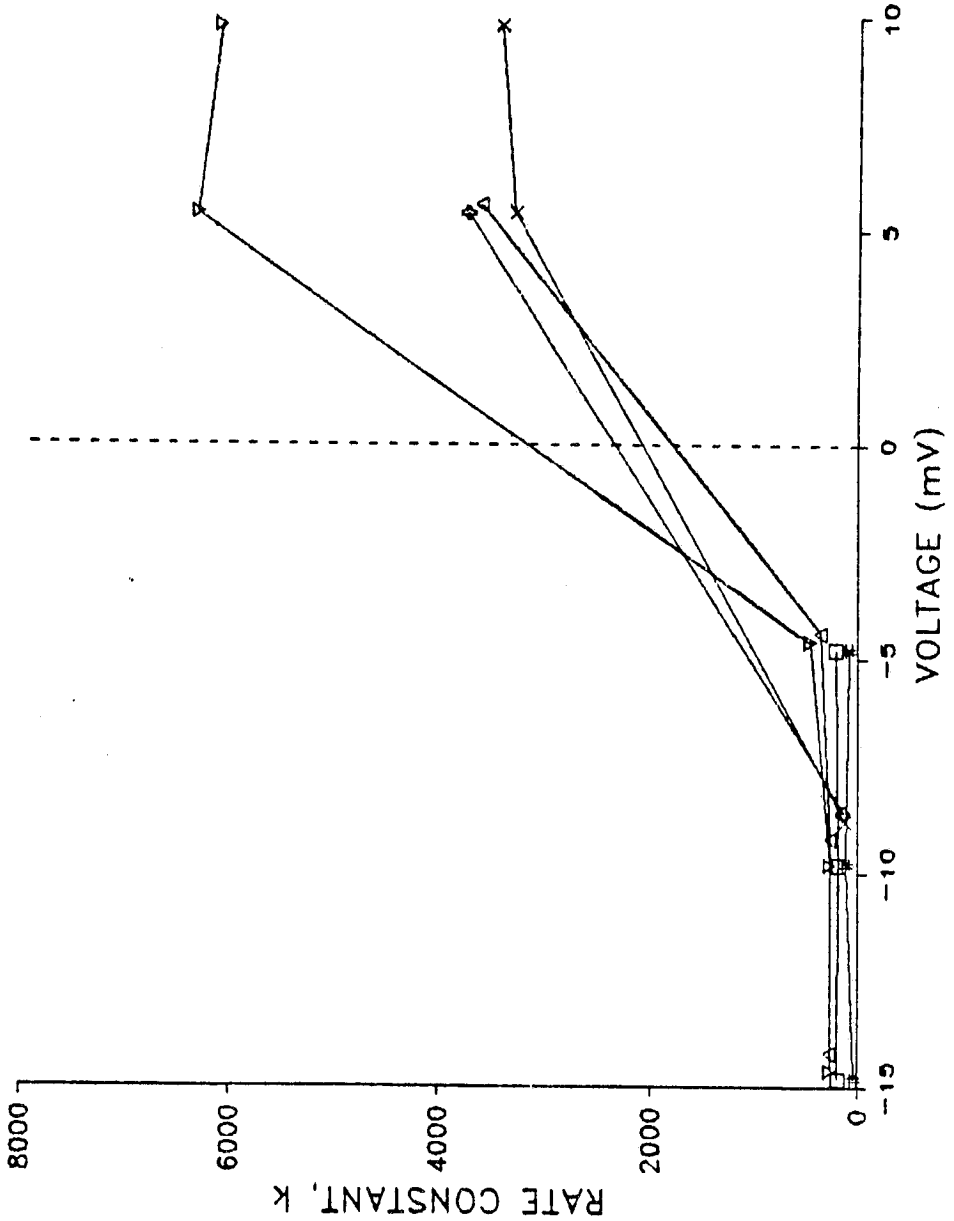

RATE CONSTANT, k
8000
6000
4000
2000
0
10
5
0
-5
-10
-15
VOLTAGE (mV)

Figure 21. Linearized rate constants of VDAC channel opening as a function of applied voltage. Log transformation of data presented in Figure 20 (averaged when replicates were available). No dependence of the opening rate constants on voltage could be detected. However, since the voltage dependence of channel closure does not account for all the voltage dependence observed in the steady state, a small voltage dependence of the channel opening rate constants was expected. Lines with slopes consistent with the slight voltage dependence expected for the opening rate constants have been drawn through 2 experiments.

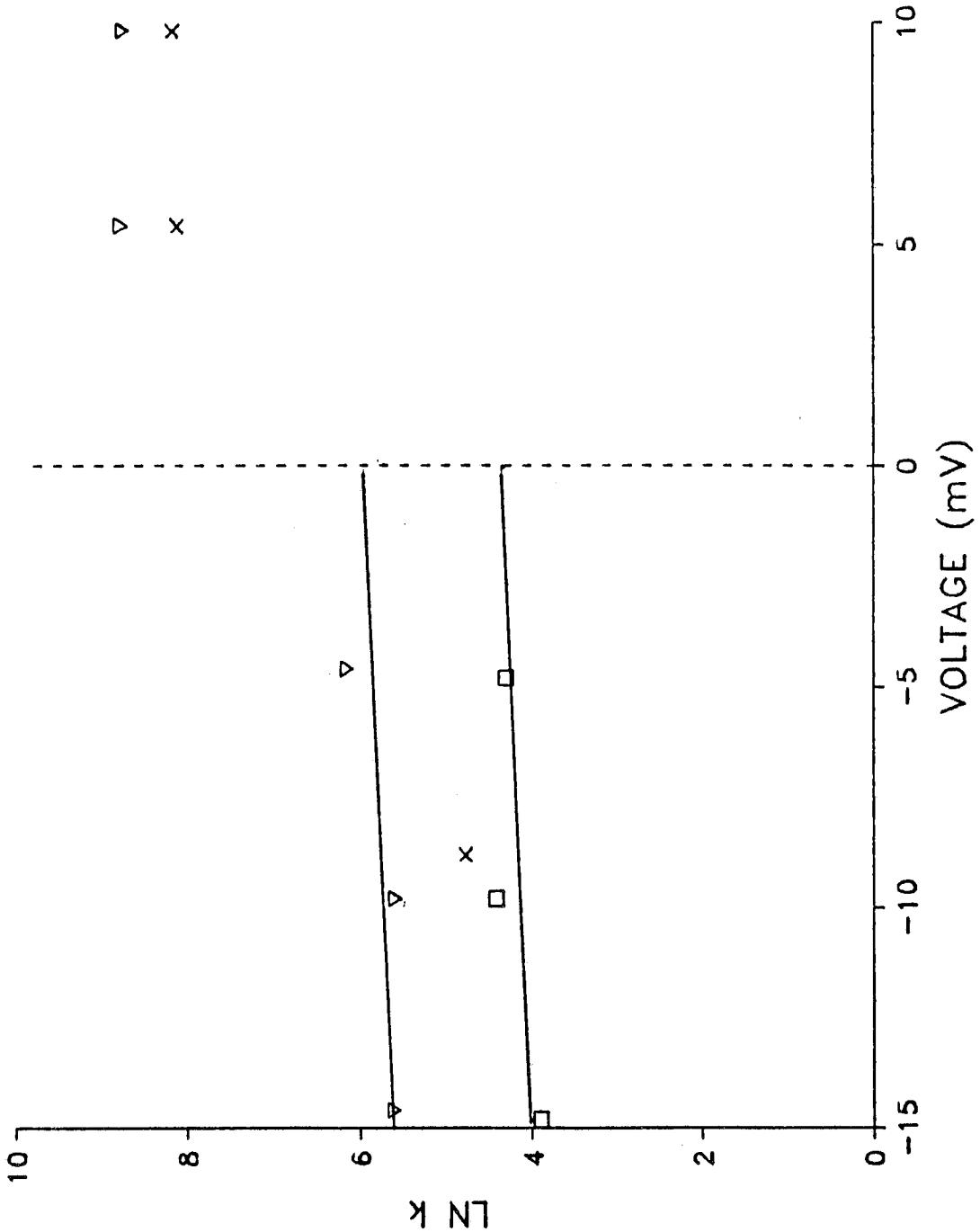
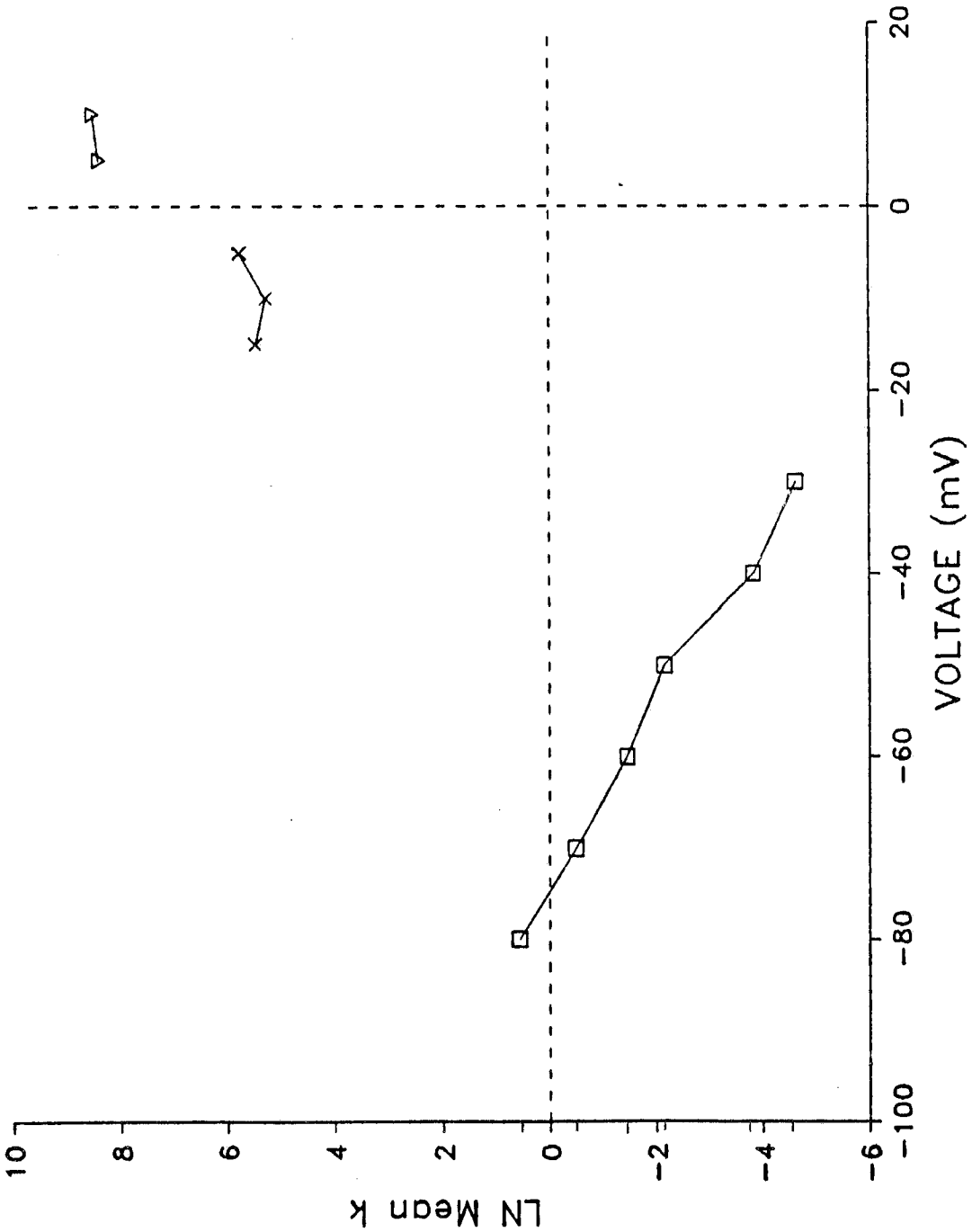

LN k
10
8
6
4
2
0
-15
-10
-5
0
5
10
VOLTAGE (mV)

Figure 22. Linearized mean rate constants of VDAC channel opening and closing as a function of applied potential: closing (□ , n=5); opening (✕, n=3; ▽ , n=2).


LN Mean k
10
8
6
4
2
0
-2
-4
-6
-100
-80
-60
-40
-20
0
20
VOLTAGE (mV)

yields a measure of the steepness of the voltage dependence of the channel closing process, 2.6. Therefore, most of the voltage dependence of the VDAC channel rests in the closing rate constant. From these experiments the opening rate constant makes no apparent contribution to the steady state voltage dependence of the channel. However, based on the closing rate constants, a small contribution of 0.6 would be estimated. Our data are not inconsistent with this small voltage dependence of the opening rate constant. Given the high variability between experiments, a shallow voltage dependence may simply not be detectable. Lines with slopes consistent with this weak voltage dependence were drawn through the data in Figure 21.

Using Eyring rate theory, the height of the energy barrier to channel closure was estimated. At -30 mV the energy barrier of closure was 83.4 kJ/mol and decreased to 70.7 kJ/mol at -80 mV. By extrapolation the potential energy barrier to closure in the absence of an applied field was 91 kJ/mol.

DISCUSSION

The kinetic analysis just presented is an attempt to probe the mechanism by which VDAC channels are voltage-gated by gaining information about the voltage dependence of the individual steps of the process. The voltage dependence of the probability of a channel being open must be reflected by voltage-dependent reaction steps. Analysis of the voltage dependence of the

closing rate constants indicates that channel closing can account for essentially all of the observed voltage dependence of the channels in the steady state. However, since the steepness of the steady state voltage dependence is 3.2 and the closure voltage dependence was 2.6, close to 20% of the steady state voltage dependence should be contributed by the channel opening process.

The voltage dependence of the opening rate constant is low. Despite the uncertainty in the estimates of the opening rate constant, the results are consistent with a weak voltage dependence of 0.6. Clearly, more experiments are needed in order to confirm the existence of a weak voltage dependence. However, these experiments are very difficult to perform and have inherent conditions which increase the error of the results (rapid kinetics that overlap with the capacitive transient; low signal levels due to the small driving force for ion flow; narrow voltage range over that the rate constants can be measured; membrane-to-membrane variability in the opening rates)

Certainly, the most intriguing observation from this study is the apparent sharp discontinuity between the rate of channel opening at positive and at negative potentials. Very rapid rates of channel opening were expected. However, the opening rate constants at positive potentials were more than an order of magnitude greater than the, already fast, opening at negative potentials. The sharp increase occurred between the 10 mV change from -5 to +5 mV. Obviously, the difference cannot be due to the

magnitude of the drop since a similar 10 mV difference between -15 and -5 mV showed no such change. The voltage dependence of the rate constant in the region between -5 and +5 mV exceeds that of the steady-state voltage dependence. The unique aspect of this region is the fact that the electric field is reversed (i.e. the potential goes from a negative to a positive value). One possible way to account for such an abrupt change in the channel's behavior between positive and negative potentials is to consider that a dipole in the molecule reorients and that this reorientation facilitates channel opening. Such a dipole(s) would most likely be large since it would have to be sensitive to very low electric fields. It is not clear whether this postulated dipole reorientation has any bearing on VDAC's voltage dependence. Since the discontinuity occurs at a potential (close to zero), which is quite distant from the switching region (the voltage region in which channels go from being mainly open to mainly closed: 10 to 20 mV), it is quite probable that this transition does not stabilize either the open or closed state.

The two state model of voltage-dependent channels predicts that there exists some electric field magnitude whose added energy to the protein would just compensate for the inherent energy difference between the states. At that applied voltage the the energy barrier between the states is equal, the rate of channel opening and closing would be equal and the probability of the channels being open or closed would be 0.5. This voltage, V_o, was obtained from steady state analysis and equaled -24 mV.

Therefore, the opening and closing rate constants should be comparable in the region of V_o. No such trend is evident in these studies. Instead, there is a marked discontinuity.

The dramatic discontinuity between the opening and closing rates could conceivably indicate that the channel opening and channel closing processes occur via different molecular pathways. Normally, in the type of analysis employed here one assumes that the two states are formed by the same sequence of molecular events and differ only in the direction in which they occur. This does not appear to be the case for VDAC. The possibility of the use of different pathways could be tested by pulsing closed VDAC channels to opening-inducing fields for very brief periods of time and looking for changes in the closure rates.

The existence of multiple, closed-channel states has been well established by two lines of evidence. First, the multi-exponential nature of the conductance drop seen during channel closure certainly points to multiple states. Second, a variety of lower conducting "closed" states can actually be observed when a single channel, inserted into planar phospholipid membranes, responds to a closure-inducing voltage (Colombini, 1986). However, the number of times a channel is observed in each state differs, and there is usually a preferred closed state which is usually in the middle of the observed range. A similar pattern is evident if the behavior of many individual channels is analyzed. However, the dependence of the closing rate constant on the amount of time channels have been held in the open state

is the first experimental evidence for the existence of multiple open states. Furthermore, the initial open channel conformation assumed by the channels is less able to respond to an applied electric field than later states. Since the closure rate constant is initially reduced, the potential energy of some or all the channel proteins in these "early" open states is greater than that of the channels after some time. Thus, the changes are most likely relaxations of the protein into states of equal conductance but at lower energy levels.

The large single channel conductance of VDAC and the relative ease with which they can be observed in the planar phospholipid membrane makes these channels good candidates for kinetic analysis by single channel methods. Such approaches require that channels oscillate between states at a rapid rate, as compared to the lifetime of the membrane, so that many events can be recorded. In addition, the properties of the channels must be such that no long-term changes in their properties occur. Efforts in our laboratory to use such an approach with VDAC were unsuccessful. The channels oscillated at very slow rates and there were long-term changes in the properties of channels. The channel displayed a kind of "memory" whereby its previous history determined future behavior. The presence of multiple open and closed states may account for the "memory" phenomenon. For example, while a channel may indeed be in a higher conducting state associated with being "open", the molecular conformation of that state is not such that it can favorably respond to an

applied field. Since that conformation was achieved as a result of the change from one state to another, the channel's conformation contains "information" about the state it has most recently occupied. Only when that conformation changes is that information lost and new memory established.

VDAC channels from N. crassa often insert into the bilayer membrane as single or double channels but quite frequently they insert in groups of three and six. Studies of negatively stained outer membranes of N. crassa have shown that the channels are present in hexagonal arrays (Mannella et al., 1983). These findings have suggested that there may be cooperativity between VDAC channels during channel closure and opening. However, channels that insert into the membrane as multiple-channel units appear to behave independently of each other after insertion. At the macroscopic level then, the channels do not exhibit cooperativity. However, these studies indicate that even at the kinetic level the VDAC channels function independently. If cooperativity between/among channels occurs, then it would be reflected in a correlation between the number of channels in a given membrane and the rate constants. No such channel correlation was observed in these studies. The rate constants of channel closure were independent of the number of channels in the membrane.

A channel protein must be subject to very subtle control in order for cells and organelles to maintain the delicate equilibrium so necessary for the effective maintenance of life.

The changes in conformation that accompany the marked changes in conductance are most likely very complex. Never the less, they must occur in response to very small environmental changes. Thus, the energy difference between the states must be very small indeed. In the case of VDAC, the estimated energy barrier from the open to the closed state is on the order of 3 or 4 hydrogen bonds (approx. 28kJ/mol). The movement of a single tyrosine residue in bovine pancreas trypsin inhibitor has an energy barrier of approximately 115kJ/mol (Karplus and McCammon, 1981).

Obtaining estimates of the rate constants of the VDAC channel opening and closing has given us estimates of the magnitudes of some of the energies involved in voltage-control of the channels. These may be extremely useful as more becomes known of the channel's structure and complex simulation studies are undertaken. However, most significantly, these kinetic studies have provided us with some provocative insights into the possible molecular mechanisms involved in voltage-gating. Future modeling must attempt to account for these complexities.

SUMMARY

1. Low levels of aluminum chloride (>1 μM) inhibited the voltage-dependent closure of VDAC channels.

2. VDAC channels could close in the presence of 100 μM aluminum but higher potentials were required to do so.

3. Single channel open conductances were uneffected by aluminum chloride levels as high as 100 μM (2.8 nS +/-0.3). The mean closed channel conductances in the absence and presence of aluminum were not significantly different; 1.0 nS +/- 0.3 and 0.8 nS +/-0.5 respectively. However, there was an apparent shift toward lower conducting closed states in the presence of aluminum. Therefore, the observed inhibition does not result from increased closed channel conductances or an inability of the channels to assume the closed state.

4. Aluminum decreased the steepness of voltage-dependence (n) and increased the voltage needed to close half the channels (V_o). The energy difference between the open and closed states in the absence of a voltage was not altered by aluminum levels as high as 100 μM. Thus, aluminum appears to interact with the voltage-sensing mechanism of the VDAC channel.

5. Aluminum (100 μM) did not affect the ability of the open channels to select anions over cations.

6. Aluminum inhibition was observed at pH 7 but not at pH 4 and the effect could be reversed by altering the pH of the bathing solution. Aluminate and aluminum hydroxide are the dominant aluminum species present in aqueous solution at pH 7 and thus, either, or both, are most likely the forms which interact with the VDAC voltage sensor.

7. Channel closing rate constants increased exponentially with increased negative applied potentials, from 0.01/sec at -30 mV to 1.77/sec at -80 mV. Analysis of these data indicated that channel closure accounts for at least 80% of the voltage dependence observed in the steady state.

8. Short periods in the open state (4-6 min) decreased the rate constants of channel closure at -60 mV suggesting the possible existance of at least 2 open states.

9. Channel opening rate constants were at least an order of magnitude faster than closure rates and had no marked voltage-dependence between -15 and -5 mV or between +10 and +5 mV. However, the rate increased an order of magnitude between -5 and +5 mV. Such a dramatic discontinuity in the rates around zero potential suggests the possible involvement of a dipole in the opening process.

10. Graphic analysis of the closing and opening rate constants reveals a marked discontinuity between the opening and closing rate constants. This would not be expected if channel opening

and closing occured via the same pathway but in opposite directions. Therefore, these processes may occur via different pathways.

11. As a result of these findings, future models of the action of the VDAC channel and research must consider these new complexities: distinct groups of charges are responsible for the voltage-gating function and the ion selectivity functions of the channel; the charges responsible for channel voltage dependence are probably located outside the channel proper; channel opening may involve a large dipole; and opening and closing may occur via different molecular pathways.

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