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Group IIIA-metal hydroxides indirectly neutralize the voltage sensor of the voltage-dependent mitochondrial channel, VDAC, by interacting with a dynamic binding site

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The voltage-dependent, anion-selective mitochondrial channel, VDAC, undergoes two different conformational changes from the open to a closed state under positive and negative applied electric fields. Micromolar quantities of aluminum hydroxide and other metal trihydroxides have recently been shown to be able to inhibit this voltage-dependent closure (Dill et al. (1987) J. Membr. Biol. 99, 187–196; Zhang and Colombini (1989) Biochim. Biophys. Acta 991, 68–78). It was suggested that the inhibition results from the neutralization of the positively charged voltage sensors by the metal species. In the present study, the dynamics of the metal-binding site accompanying channel closure was investigated by adding $In(OH)_3$ to only one side of the membrane and examining its effect on the channel's gating processes. Indium added to open channels inhibited channel closure only when the metal-containing side was on the lower potential side of the applied field. If indium was added only to the higher-potential side, the channels closed and tended to remain closed after the field was abolished. The addition of metal hydroxide after closing the channels with a negative potential on the metal side did not result in channel re-opening as would be expected for sensor neutralization. Inhibition occurred immediately, however, if the channels were first allowed to open briefly. The closed-state selectivity seemed to be very similar in the absence or presence of the metal, indicating that the metal-binding sites are not located within the pore of the channel in the closed conformation. The results are consistent with a voltage-dependent translocation across the membrane of each of two metal-binding sites on VDAC. This translocation is tightly coupled with channel opening and closing.

Introduction

VDAC channels are the pathways in the mitochondrial outer membrane through which much of the molecular traffic between the cytoplasm and the mitochondrial spaces is thought to flow. VDAC channels have been found and studied in a wide variety of organisms ranging from protists to mammals [1–11]. Two important, source-independent properties of these channels are their voltage dependence and anionic selectivity [12].

To understand the nature of the voltage dependence of the rat-liver VDAC channel, Bowen and co-workers [13] investigated the relationship between the voltage dependence and the pH of the solutions bathing the VDAC-containing phospholipid membranes. By raising the pH they were able to reduce the channel's voltage dependence in a manner consistent with the neutralization of the voltage sensor. The apparent pK of the titratable entity was 10.6, consistent with that of lysine ϵ-amino groups. While arginine modifiers [13] had no effect on the voltage dependence of VDAC, the amino group modifier [14], succinic anhydride, was shown to reduce the channel's voltage dependence and reverse the ion selectivity simultaneously [15,16]. These observations indicate that VDAC's voltage sensor contains amino groups and the motion of these charges through the electric field is responsible for the channel's voltage dependence. A model was proposed in which one set of positive charges located along the inner wall of the channel is responsible for both the voltage dependence and anion selectivity of the channel [17]. However, no unambiguous link between the two has been established.

This model was challenged by later studies with aluminum [18]. Aluminum was shown to reduce the

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channel's voltage dependence without changing the open-channel selectivity. This finding suggests that the sensor and the selectivity filter are two separate entities and that the sensor is, most likely, located outside the pore of the channel [18].

A more recent study has shown that other metals, including indium, gallium, and a few trivalent transition metals, are also capable of inhibiting channel closure [19]. The active form of the metals were shown to be their neutral trihydroxylated species, as opposed to the usually-expected M^{3+} [19]. This indicates that the inhibition is probably not achieved by direct electrostatic binding between the metal and the charged groups on the sensor.

In the present study, the dynamics of the voltage-dependent conformational changes of VDAC were investigated by asymmetrical addition of a metal trihydroxide and by monitoring the ion selectivity of the channel in the closed state. The results have provided unexpected information towards a better understanding of the voltage gating process of VDAC.

Methods

Preparation of channels

As described by Mannella [20], mitochondrial membranes were isolated from log-phase suspension cultures of a wall-less mutant of *Neurospora crassa* (ATCC No. 32360). Isolated mitochondria were lysed by suspending in a hypoosmotic solution, and the membranes were pelleted at $25000 \times g$ for 30 min at 4°C. Finally, the membrane pellets were suspended in a solution containing 1 mM KCl, 1 mM Tris-HCl (pH 7.4) and 15% (v/v) DMSO to a final protein concentration of approx. 3 mg/ml. The suspension was stored in aliquots at −70°C.

Experimental procedures and data analysis

All experiments were performed on planar phospholipid membranes generated according to the method of Montal and Mueller [21] with modifications [1]. Membranes were made across a 0.1 mm-diameter hole in a Saran partition separating two buffered salt solutions. Soybean phospholipids [1] were used to make the membranes. For the experiments involving asymmetrical additions of $InCl_3$ the two solutions were identical, each containing 1 mM KCl, 5 mM $CaCl_2$, and 10 mM Bistris (pH 7.0). In the second set of experiments, the KCl concentration of one solution (the trans side, as defined below) was only 0.1 M, giving rise to a 10-fold salt gradient. The pH was 5.0 and gallium instead of indium was used *.

An operational amplifier was used to clamp the transmembrane potential, and the membrane current was recorded on a Kipp & Zonen BD41 chart recorder [1]. The solution held at virtual ground by the amplifier is defined as the trans side so that the signs of potentials refer to those of the solution on the other side of the membrane, the cis side.

Before use, an aliquot of the VDAC preparation was thawed and diluted thrice with distilled water to assure that only moderate numbers of channels were added to the bathing solution. Triton X-100 was added to a final concentration of 1% (v/v), and the mixture was let stand at room temperature for at least 10 min prior to use. After formation of the membrane, 5–10 μl of the VDAC dilution was added to the solution on one side of the membrane (usually the cis side unless otherwise indicated). The solutions were stirred during the addition of VDAC, and a low potential was applied (−10 mV) in order to monitor the changes in membrane conductance. The insertions of individual channels were monitored and seen as stepwise increases in current.

High purity (> 99.9%) gallium chloride and indium hydroxide were obtained from Johnson Mathey Inc., and the $In(OH)_3$ was converted to the chloride salt by adding HCl. These metal-chloride solutions were acidic but in the presence of 10 mM buffers the small quantities added (2–6 μM final concentrations) did not result in pH shifts greater than 0.2 units.

Calculation of the closed-state reversal potential

Current-voltage curves recorded from membranes containing a few channels (four to six) were used as the source of information. Reversal potentials of the control channels were obtained by extrapolating to zero current the portion of the curve where all channels were closed. For gallium-treated channels, the level of gallium was chosen to ensure sufficient inhibition so that only one of the channels would close. The conductance change associated with that closing event was determined from the change in slope of the *I-V* curve. The reversal potential of that channel was determined as follows:

$$I' = ng_o(V - E_o) + mg_c(V - E_c) \quad (1)$$

$$I = (n-1)g_o(V - E_o) + (m+1)g_c(V - E_c) \quad (2)$$

* In the present experiments a pH of 5.0 was used because in the literature on VDAC most selectivity measurements were obtained under non-buffered conditions (pH ≈ 5). $InCl_3$ was first used and was soon found to make the open channel more anion selective than usual. $AlCl_3$ was then tested and the same effect was observed. This was unexpected because aluminum had been shown not to affect the open-state ion selectivity at neutral pH [18]. To be certain that these observations were not due to experimental error, both metals were tested again at pH 7.0 and both had no effect on ion selectivity. It is very likely that at pH 5 another species (besides $M(OH)_3$), possibly M^{3+} is involved and interacts with the selectivity filter. The observation that $GaCl_3$, when later tested, had no effect on selectivity at pH 5.0 supports the above hypothesis because both the $[In^{3+}]$ and the $[Al^{3+}]$ are fairly high at pH 5.0 while the $[Ga^{3+}]$ is very low [19]. To avoid complications and assure that the interaction between the metal and the gating mechanism could be unambiguously studied, pH 5.0 and gallium were employed in the ion gradient experiments.

where I' and I are the current levels prior to and following the closure of one channel, respectively; V is the transmembrane potential; n, g_o, and E_o are, respectively, the number, single-channel conductance, and reversal potential of the open channels in the same membrane; m, g_c, and E_c are those of the closed channel. When $I = 0$, by definition $V = E_{n-1}$, the average reversal potential of the membrane with channels being open and one channel closed. Since E_{n-1} and E_o can be directly, unambiguously measured from the I-V curves when one channel is closed and when all are open, respectively, Eqn. 2 can be rearranged to calculate the closed-state reversal potential:

$$E_c = [(n-1)g_o(E_{n-1} - E_o) + (m+1)g_c E_{n-1}]/(m+1)g_c \quad (3)$$

Some E_c values of control channels (without $GaCl_3$) were also obtained this way in order to be certain that the difference, if any, between the two groups (control and treated) had not resulted from the use of different methods of estimation.

Nomenclature

The potential on the trans side was held at virtual ground and defined as zero. The signs of the potentials mentioned in this paper therefore always refer to those of the cis side relative to zero, the trans side potential. Unless otherwise indicated, the channels were always added to the cis side, and the metals were added either to the trans side only (asymmetrical addition), or to both sides (ion gradient experiments). In the text and figure legends regarding the 'asymmetrical addition' experiments, the sign and magnitude of a potential is often followed by a statement (in parentheses) which serves to indicate whether the metal was on the lower or higher potential side. For example, at -40 mV applied potential, the metal, which was added to the trans side, was at the higher potential side ($0 > -40$). This is necessary because the sidedness of the metal's effect on the voltage dependence of the channel is the major issue addressed in this paper (see Discussion).

Results

The dynamics of the metal hydroxide-binding site on VDAC, i.e., the translocations of the binding site accompanying the conformational changes of the channel, were explored in two ways: (i) by adding the metal hydroxide to the solution on one side of the membrane and examining its effect on the channel's gating processes; (ii) by analyzing the characteristics of the channel's closed-state selectivity in the presence of the metal hydroxide. Indium, a more effective inhibitor than aluminum [19], was used for the first part of the study. In the selectivity experiments gallium and pH 5 were used.

Neurospora crassa VDAC channels were inserted into planar phospholipid membranes by adding detergent-solubilized VDAC to the cis side of the membrane (unless otherwise indicated). The two aqueous bathing solutions each contained 1 M KCl, 5 mM $CaCl_2$, and 10 mM Bistris (pH 7.0). After a relatively steady membrane current level had been achieved under a low-voltage driving force (usually -10 mV), the channel's voltage dependence was tested by applying positive and negative closing potentials (usually higher than ± 20 mV), and the channel-closure $1/e$ decay time (the time required for the current level to decrease to $1/e$ of its total decay) was used as a quantitative measure of the voltage dependence. $InCl_3$ was then added to the solution on the trans side (unless otherwise indicated), and the same characterization procedure was repeated. After each experiment the pH on both sides were re-measured and no change greater than ± 0.2 units was observed.

Fig. 1 shows a portion of a recording of current flowing through VDAC channels in response to various applied potentials (signs of potentials always refer to

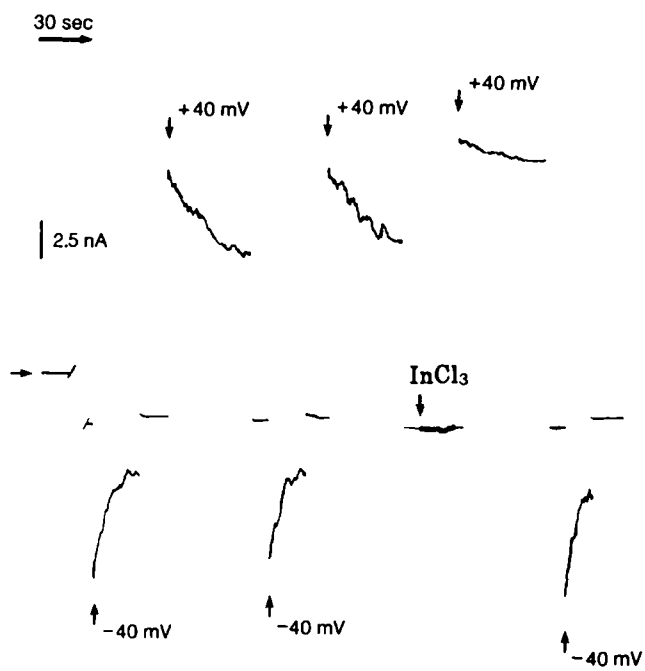

Fig 1. The addition of indium chloride to only one side of a membrane results in the inhibition of only one gating process. Initially both aqueous solutions contained 1 M KCl, 5 mM $CaCl_2$, and 10 mM Bis-Tris (pH 7). VDAC channels were inserted by adding an aliquot of the detergent-solubilized sample to the solution on the cis side. The sign of the membrane potential always refers to the cis compartment, the trans side was held at virtual ground. At the indicated points (vertical arrows) voltage-dependent channel closure was tested under ± 40 mV applied potentials, and the resultant transmembrane current is shown. After each pulsing the potential was returned to -10 mV to allow the channels to re-open. Indium chloride was then added only to the trans side, to a final concentration of 2 μM. Note that the metal was on the lower potential side under positive potentials. Channel closure was tested again by applying +40 or -40 mV. The horizontal arrow indicates the zero current level.

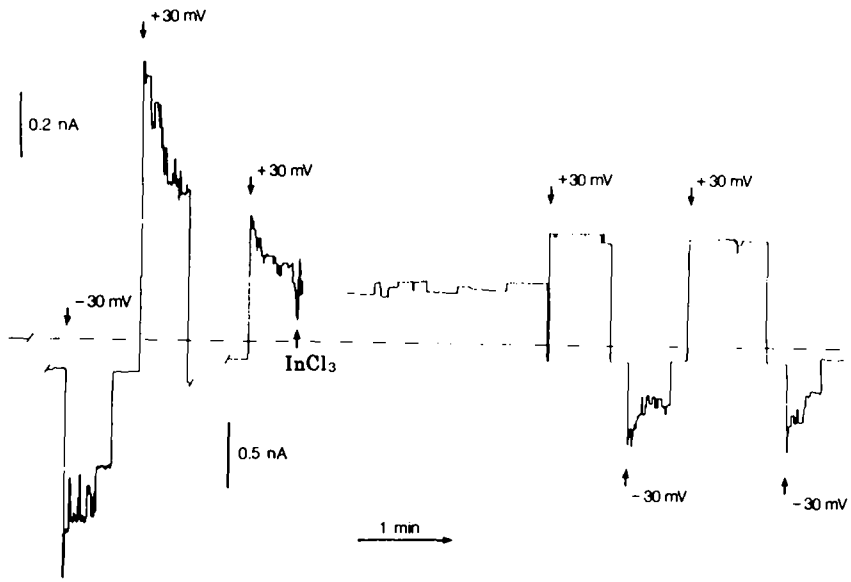

Fig 2. Effect of indium on channel closure when added with channels closed. Experimental conditions and procedures were essentially identical to those described in Fig. 1, except that the channels were held in the closed state at the time of metal addition (2 μM final concn.) and during the first 2 min after the addition. Note the scale change after the first two pulses of the experiment. The sign convention for the applied potentials is the same as in Fig. 1 and note that, between pulses at the indicated voltage, the potential was returned to -10 mV.

the cis side). Prior to the addition of $InCl_3$, rapid channel closure was observed at both $+40$ and -40 mV as indicated by the steep current decay following the application of the potentials (the residual current is that flowing through the low-conducting, 'closed-state' channels). Following the addition of 2 μM $InCl_3$ to the trans side, closure under positive potentials (the metal-containing side was the lower potential side) was markedly inhibited while that under negative potentials was not affected (also see Fig. 2). In 13 different experiments in which indium was added only to the trans side, closure at positive potentials was either too poor to measure a 1/e decay time or had an increase in 1/e decay time between 3- and 11-fold. In all cases, closure under negative potentials was not significantly affected with a fold change in 1/e decay time after indium addition of 0.9 ± 0.2. The complementary experiment, VDAC added to the trans side and $InCl_3$ to the cis side, resulted in inhibition of closure at negative potentials while that at positive potentials was not affected (not shown). It should be noted that in the experiment (Fig. 1) illustrated, the closure rates of the control channels appear to be greater under negative potentials than under positive ones. This was often observed and does not affect the interpretation of the results *.

Channel closure prior to indium addition appeared to protect the channels from the action of indium. As shown in Fig. 2, channels were closed prior to indium addition by applying a 30 mV potential. This potential was maintained for about 2 min following the addition of $InCl_3$ (2 μM final concn.) to the trans side. During the entire 2 min little if any channel opening was detected. However, after the membrane potential was dropped to -10 mV for less than one second to allow the channels to open, the immediate re-application of a 30 mV positive potential failed to close the channels. Thus, sensitivity to indium was restored by a brief period of time in the open state.

While indium addition to the trans side inhibited voltage-dependent channel closure under positive and not negative potentials, once the channels were closed with negative potentials, their opening rates were markedly reduced by the presence of indium. Under negative potentials the channels seemed to be free to close but tended to stay closed after the potential had been decreased to -10 mV (Fig. 3). This phenomenon was highly reproducible and quantitatively significant. To estimate the extent to which channels stayed closed after having been closed under a negative potential (metal on the higher potential side), membranes containing only a few (five to twelve) channels were used so that the exact number of channels could be directly measured and current-change events precisely understood. The results were expressed as 'the number of channels that stayed closed as a percentage of total channels closed'. As summarized in Fig. 4, the percentages of channels that stayed closed after a negative potential were quite high whereas those in response to a positive potential were negligible.

* Since the 1/e decay times after indium addition were always compared to the controls obtained prior to metal addition, the variability in the control 1/e decay times should not affect the interpretation of the results. Moreover, the faster rate of channel closure under positive potentials was observed whether VDAC was added to the cis or trans side, indicating that it may have been due to an inherent asymmetry in the lipid bilayer.

Fig 3. Tendency of the indium-affected channels to stay closed after being closed under negative potentials. Experimental conditions and procedures were identical to those described in the legend of Fig. 1. In the presence of 2 μM $InCl_3$ (added to the trans side) the channels remained closed (the large arrows) after the −40 mV voltage pulse. Signs convention of the applied potentials is as indicated in Fig. 1. The metal was on the higher potential side under negative potentials.

Although it was shown that aluminum addition did not change open-channel selectivity [18], the possibility exists that the metal binding site moves into the channel upon channel closure. If metal binding were to alter the charge (number or sign) within the pore in a closed state, this change could be detected as a closed-state selectivity change with respect to that of a control channel. The experiments were performed, at pH 5, on membranes containing only a few (no more than six) channels in the presence of a salt gradient (1 M vs. 0.1 M KCl). After insertion of the channels, an asymmetric triangular voltage wave was applied and the transmem-

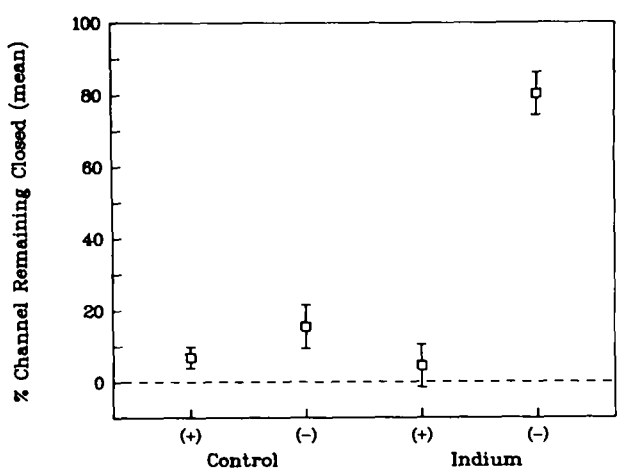

Fig. 4. Summary of many observations similar to the one illustrated in Fig. 3. Shown are the mean values (% channel remaining closed) and their standard errors obtained from membranes containing control or indium-affected channels. Symbols below the X-axis refer to the signs of potentials under which the data had been obtained and refer to the potential on the cis side while indium was added to the trans site. The number of observations included in the figure are: 17, 13, 10, and 12 for control (+), control (−), indium (+), and indium (−), respectively.

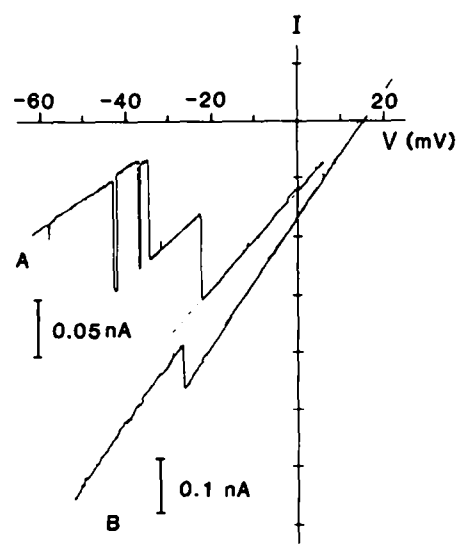

Fig 5. A current-voltage plot recorded in the presence and absence of $GaCl_3$. Experiments were performed on membranes with only a few channels in the presence of a 10-fold salt gradient (1 M vs. 0.1 M KCl). After insertion of the channels, an asymmetric triangular voltage wave was applied travelling between −64 and +22 mV at a rate of 0.7 mV/s, and the transmembrane current was simultaneously recorded. I-V curves in the absence (line A) or presence (line B) of 16 μM $GaCl_3$ were obtained from the records when the voltage wave was travelling from negative to positive potentials. Open-channel reversal potentials were measured by extrapolating the current tracings to $I = 0$ when all channels were open (intercept of the line on the X-axis). Closed-channel reversal potentials were obtained by extrapolation and calculation based on the events in which only one channel was closed.

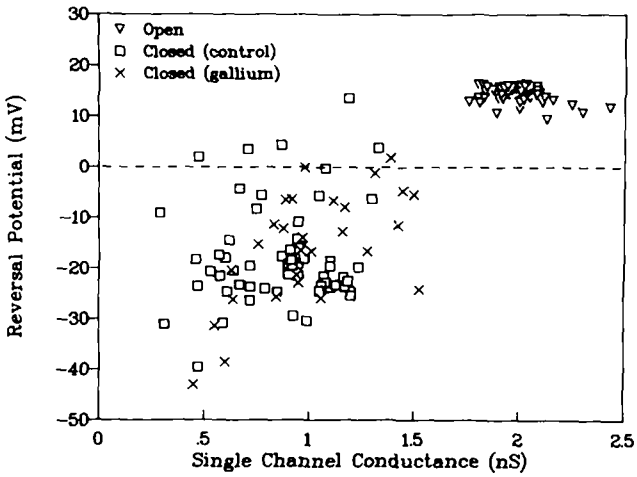

Fig 6. Conductance and reversal potential for channels in the open and closed state with and without gallium present. These results were obtained from experiments such as the one illustrated in Fig. 5. Open- or closed-state reversal potentials in the absence or presence of $GaCl_3$ are plotted against the corresponding single-channel conductances.

brane current was simultaneously recorded (Fig. 5, line A). Gallium chloride was then added to both sides of the membrane, and the *I-V* curve was again recorded (Fig. 5, line B). Open-channel reversal potentials were directly measured by extrapolating the current tracings to $I = 0$ when all channels were open (Fig. 5). Since strong inhibition of channel closure must be present when evaluating the channel's selectivity characteristics, closed-channel reversal potentials had to be obtained by both extrapolation and calculation based on the events in which only one channel was closed (see Methods). Fig. 5 shows that after the addition of 16 μM $GaCl_3$ channel closure became a rare event while the open-state reversal potential did not change as evidenced by the same intercept of the two lines, A and B, on the voltage axis. The results from several experiments are summarized in Fig. 6. While the open-channel reversal potentials and conductances all fall into a narrow range, there are apparently multiple closed states as demonstrated by the much wider distribution of both the closed-channel reversal potentials and the closed single-channel conductances. However, the overall distribution patterns of the closed-state reversal potentials of the gallium-treated (×) and the control (□) channels are very similar.

Discussion

It was clear when VDAC was first discovered in 1975 [1] that each VDAC channel can undergo two voltage-dependent conformational changes, one under positive potentials and the other under negative potentials. Later, evidence emerged to indicate that these changes represent two separate gating processes that result in two fundamentally different closed states [22]. The use of a chemical modifier, succinic anhydride [15,16], and perturbing agents, polyanions [23] and the VDAC modulator [24], showed that each gating mechanism could be affected independently.

In the original experiments [18], which showed that aluminum was capable of inhibiting the voltage-dependent closure of VDAC, the metal salts were added to the solutions on both sides of the membrane. Even so, some asymmetry was observed and was attributed to asymmetrical free metal concentrations caused by adding VDAC and detergent to one side only. In the present study, asymmetrical additions of indium salt resulted in selective inhibition of only one of the two gating processes. In the presence of indium, normal channel closure only occurred when indium was on the higher-potential side (under negative potentials), and the closure in response to positive potentials (indium on the lower-potential side), was greatly inhibited.

The present results have provided evidence to show that the indium-binding site is translocated through the membrane during the conformational change from open to closed state (see Fig. 7). While readily undergoing voltage-dependent closure when the indium side was made positive, the channels, once closed, tended to remain closed. The simplest explanation for this phenomenon would be that the indium-binding site that had been hidden from indium in the open state traversed the membrane during the closure process and became now exposed to the indium-containing medium; the subsequent binding by indium hydroxide probably stabilized the closed state. Additional supporting information comes from the type of experiment shown in Fig. 2. That the addition of indium to channels held closed by positive potentials (negative on the indium side) did not result in channel opening and that a transient re-opening of the channels resulted in inhibition of channel closure indicate that the indium-binding site that was accessible to indium became hidden when the channels were closed.

An alternative hypothesis to the translocation of the metal binding site through the membrane is that the site merely becomes more or less able to bind indium depending on the channel's conformation. If so, then *each* portion of the channel responsible for *one* of the two gating processes must have two indium binding sites, one on the cis and the other on the trans side of the membrane *. One site, exposed in the open state, binds indium and inhibits channel closure and the other, exposed in the closed state, inhibits channel opening. Only in this way can one explain the finding that if indium added to one side inhibits channel closure due

* The possibility of indium diffusing through the channel and acting on a site on the other side is remote because the indium-free compartment acts as an infinite sink and because we would not be observing asymmetrical effects if this process were significant.

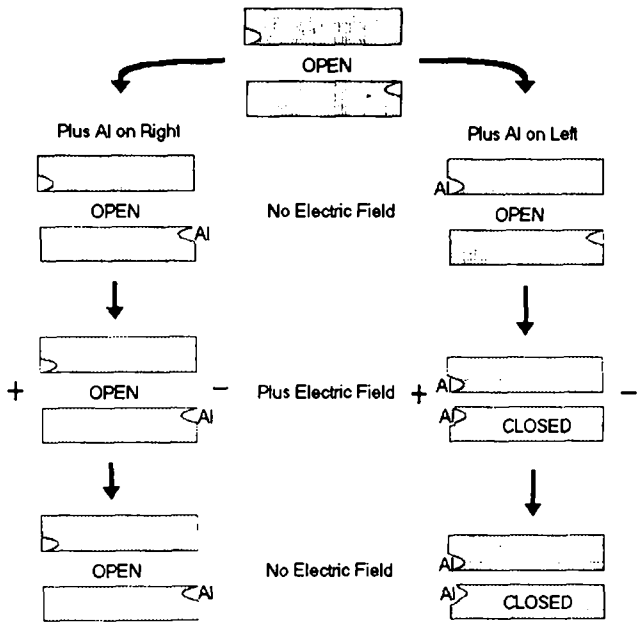

Fig 7. Schematic drawing of a model which embodies the observed metal-VDAC interaction. The longitudinal cross section of each cylindrical channel is represented by two rectangles, one for each subunit, and the space between the two rectangles represents the pore of the channel. Small notches on the rectangles represent the metal-binding sites. At the top of the figure, aluminum is added to different sides of the channel in the absence of applied potential (therefore channels are open). An electric field is then applied so that in one situation (left) the aluminum is on the lower-potential side and in the other (right) it is on the higher-potential side. The channel on the left cannot close due to aluminum binding from the lower-potential side whereas the channel on the right closes. Closure results in the translocation of one site across the membrane. Both binding sites of the closed channel are now on the aluminum side and therefore aluminum can bind to both. This channel remains closed after the removal of the field, probably due to stabilization of such a conformation by aluminum binding.

to one gating process, then indium must be added to the other side to inhibit the opening rate for that *same* gating process. It seems less complex to consider one metal site per gating process which translocates through the membrane and indeed the two hypotheses are functionally equivalent.

The possibility that the indium-binding site does not move all the way across the membrane and becomes lodged within the channel's aqueous pore, was tested by examining the channel's closed-state selectivity in the presence of indium. The previous finding that aluminum did not affect the open-state ion selectivity [18] suggests that the metal-binding site was located outside VDAC's pore. However, upon channel closure the binding site could conceivably be moved into the pore. Since it is likely that metal binding results in a change in charge at the binding site (in order to account for the metal's inhibitory effect on voltage gating), movement of the site into the pore would be expected to alter the ion selectivity of the closed state. However, despite the variabilities in the measurements of the closed-state selectivity, the present experiments have shown that the overall distributions of the values obtained in the absence or presence of indium appear to be very similar (see Fig. 6). Therefore the results indicate that the metal, while inhibiting channel closure, does not affect the closed-state selectivity, indicating that the translocation of the binding site is fully across the membrane.

If the metal binding site is indeed translocated across the membrane upon channel closure, the direction of this translocation is not consistent with the expected direction of translocation of the voltage sensor. Based on the strong evidence provided by earlier studies [13,15,17,23] that the sensor was composed of positive charges, and on their new observations, Dill and coworkers [18] strongly suggested that aluminum acts by neutralizing the voltage sensor(s). Therefore, if the voltage-dependent channel closure were coupled to the movement of positive charges through the transmembrane electric field, the closure with positive potential on the indium-containing side should be in consequence of the translocation of the positive sensor from the indium side to the opposite side. Were indium binding to directly neutralize the sensor, channel closure with positive potential on the indium side would be inhibited. Since the observed effect was the opposite to the above prediction, the indium-binding site is highly unlikely to be the sensor itself. A very different mechanism must be considered to account for these observations.

The evidence that aluminum neutralizes VDAC's voltage sensor was derived from the finding that aluminum reduced the channel's voltage dependence while increasing the voltage needed to close half the channels [18]. This mechanism should also apply to indium and other metals that have been shown to interact with VDAC in much the same way as aluminum [19]. It is possible to reconcile the observations from asymmetrical indium addition with the aluminum results by postulating that the conformational change associated with VDAC closure results from the movement of a positively-charged sensor across the membrane and also results in the translocation of an uncharged metal-binding site in the opposite direction. If indium binding were to generate a net positive charge at the binding site, then this positive charge would be translocated in the opposite direction to the movement of the sensor. This would effectively neutralize the charges on the voltage sensor and consequently negate the voltage-dependent energy change needed for the gating mechanism to function.

These findings are consistent with the current view of VDAC's molecular structure and voltage-gating mechanism [12]. Experimental evidence indicates that the functional channel is a homodimer [22] and the two subunits together form a simple cylindrical pore [25]. The subunits are proposed to be oriented in opposite

directions across the membrane, each forming half the cylinder to form a symmetrical channel [12]. In this arrangement (Fig. 7) the two monomers would respond, respectively, to negative and positive electrical fields to achieve two different closed states. Likewise, each monomer possesses one indium-binding site so that the channel, in the open state, has an indium-binding site on each membrane surface. Each binding site is structurally coupled to a sensor on the opposite side of the membrane so that when a sensor moves in the applied field, resulting in channel closure, its coupled indium-binding site is translocated across the membrane in the opposite direction. This model explains the mechanism of (i) the inhibition of channel closure, (ii) the inhibition of channel re-opening after having been closed, and (iii) the differences between the results obtained with indium present on only one side and on both sides.

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