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This publication is an invited chapter for a book dedicated to the actions of aluminum in biological systems. This contribution focuses on the effects of aluminum on VDAC. This is a summary of results published in other journals by people in my laboratory.

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Aluminum and Membrane Channels

M. Colombini

Introduction

Cell membranes contain a variety of transport systems which control the flow of ions and small molecules from one cellular compartment to another. One such system is the channel-forming protein. This is a protein that is embedded in the membrane and forms a continuous water-filled pathway through the membrane. Such a pathway would tend to dissipate electrochemical gradients and thus is generally under some sort of control.

Gating refers to the variety of processes that exist in different membrane channels to control their permeability. Some are gated by the membrane potential (voltage gated), some by small molecules (chemical gated), and some by the tension in the membrane (stretch gated). Gating relies on the channel-forming protein existing in different conformational states differing in their ability to allow molecules and/or ions to cross the membrane. The gating process allows relatively small changes in the environment to cause dramatic shifts in the state occupied by a given channel. For example, in the case of a voltage-gated channel, the channel may have a high probability of being in an open or highly-permeable state in the absence of a membrane potential and a low probability of being in the open state in the presence of even a small potential. For these gating processes to be useful physiologically, the different conformational states must be very close in energy, often differing only by a fraction of the energy of a hydrogen bond.

Metal ions such as aluminum could, in principle, interfere with finely-tuned systems such as gated membrane channels if they bind to these proteins. This is reasonable from hindsight, but when effects of aluminum on voltage-gated channels were first observed, they were a big surprise. Indeed, they were discovered by accident.

Effects of Aluminum Salts on Membrane-Channels

Mitochondria from all eukaryotic kingdoms¹ contain channels in their outer membranes that allow ions and small molecules to diffuse between the cytoplasm and the mitochondrial spaces. These channels are called VDAC, an acronym for voltage-dependent anion-selective channel. As the name indicates, these channels respond to the membrane potential and enter closed conformations when a voltage is applied. This voltage-gating may allow these channels to control mitochondrial function by controlling the permeability of the outer membrane.

VDAC channels have a relatively simple structure. They form 3 nm diameter pores with just one 30 kDa polypeptide.² In the outer membrane of *N. crassa* they can be induced³ to form two-dimensional crystals (Fig. 1) whose structure consists of a 6-channel repeating unit. At the resolution of the images, the surface structure of the crystal seems the same from both sides reflecting the symmetrical electrophysiological behavior.

Less than 10 μ M $AlCl_3$ causes a reduction in the voltage dependence⁴ of VDAC.¹ At higher levels one observes a profound change in the ability of VDAC to respond to a membrane potential. These channels are normally open at low membrane potential and close when positive or negative voltages are applied (the switching region is around ± 20 mV).

Channel closure can be observed as a decay in ion flow through the channels as a function of time after the application of a membrane potential (Fig. 2). VDAC channels were reconstituted into planar phospholipid membranes separating two aqueous compartments. This allows the properties of the channels to be studied without the influence of other cellular components. The composition of the solution



Fig. 1. Two-dimensional crystals of VDAC channels in outer mitochondrial membranes of *N. crassa*. The left panel is an electron micrograph of freeze-dried and shadowed outer membranes after treatment with phospholipase A₂ to induce large crystalline arrays. The crystalline pattern is visible on the surface of the central flattened vesicle with straight edges. On the right are the results of computer filtration, averaging and image reconstruction. The false color indicates elevation (bright yellow being high and dark blue being low). The two images represent the two surfaces of the crystal. Each image contains four six-channel repeating units. The figure is adapted from the work of Thomas *et al.*² The color images were produced by B. L. Trus.



on either side of the membrane can be controlled exactly and so can the membrane potential $\Delta\psi$. The potential can be used to drive ions through the channels and the resulting current can be used to access the permeability of the population of channels in the membrane. If a closure-inducing membrane potential is applied, the current through the population of channels decreases with time as channels undergo conformational changes from the highly-conductive, open, state to a low-conducting, closed, state.

The rate channel closure and the extent of

closure were dramatically reduced after the addition of AlCl_3 . A remarkable aspect of these findings is that the experiments were performed at pH 7 where the concentration of free Al^{3+} is vanishingly small due to its hydration in solution to hydroxylated forms. Even more remarkable was the fact that increasing the free concentration of Al^{3+} by many orders of magnitude, by reducing the pH to 4 (keeping the total aluminum constant) essentially eliminated the effect of aluminum (Fig. 3). This indicated that the species that acts on VDAC is not Al^{3+} , but some other form of aluminum

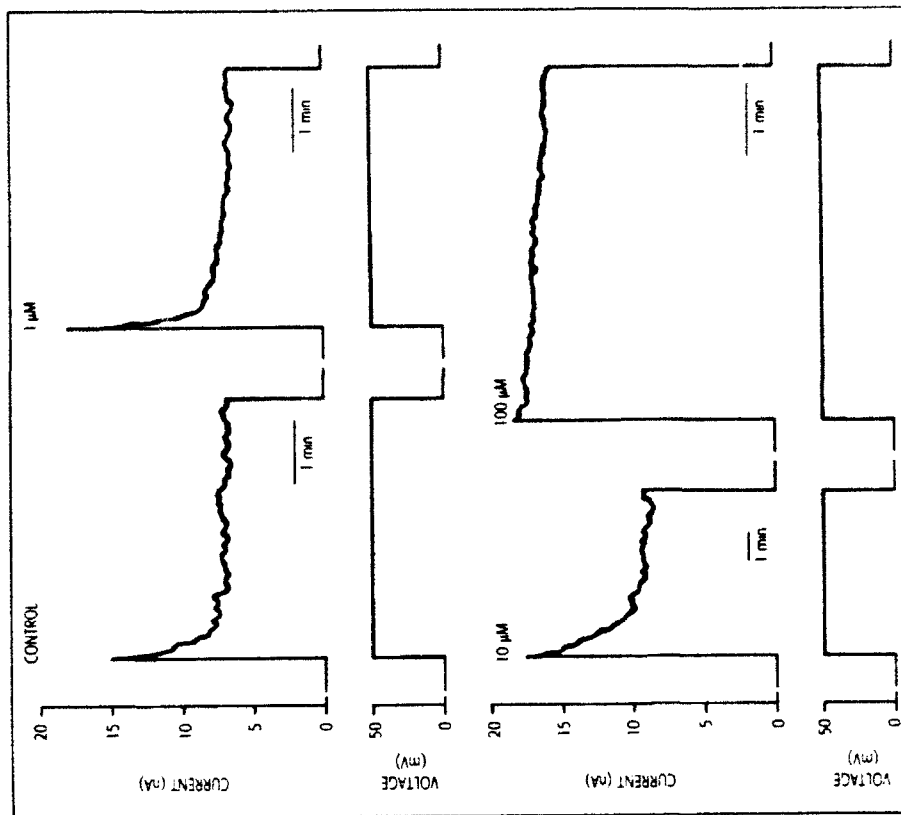


Fig. 2. Low levels of AlCl_3 decrease both the rate and extent of voltage-dependent channel closure. Sequential additions of AlCl_3 were made to a planar phospholipid membrane (soybean phospholipids) containing many VDAC channels isolated from the mitochondria of *N. crassa*. The current levels were recorded in response to the applied voltage as indicated. Reproduced with permission from Dill *et al.*⁴

more common at physiological pH.

Experiments on neuroblastoma cells⁵ show that Pb^{2+} , Cd^{2+} , and Al^{3+} additions open up channels in their surface membranes. These channels were normally closed and induced to

open by the addition of these metals. Although the authors speculate that this may be a metal ion activated channel, the results are quite similar to those obtained with VDAC. The ability of the channels to remain closed

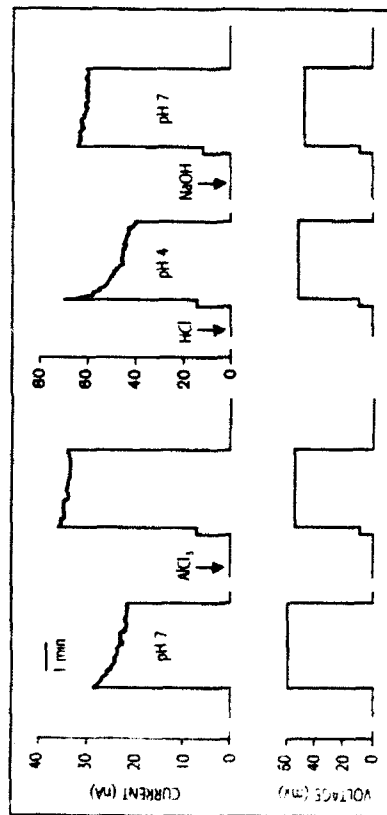


Fig. 1. Aluminum's inhibition of VDAC's voltage dependence is reversed at acid pH. VDAC channels from *N. crassa* mitochondria were inserted into a planar phospholipid membrane made using diphyanyl phosphatidylcholine. 100 μ M AlCl₃ was added to the solution on both sides of the membrane (1 M KCl, 5 mM CaCl₂, 10 mM Tris-HCl, initially pH 7). The records were all obtained in the same experiment. The breaks in the record indicate that only the relevant portions of the record are displayed. Since channels kept on setting throughout the experiment, the scale had to be changed in the middle. Reproduced with permission from Dill *et al.*⁴

under normal conditions is inhibited by the presence of aluminum. The experiments were performed at pH 7.2 indicating that a species other than Al³⁺ is probably involved but the authors did not explore this possibility.

This ability of aluminum fluoride to activate G proteins which can, in turn, affect membrane channels, has been established and will not be discussed here because the high levels of fluoride needed are not physiological.

Aluminum Hydroxide as an Active Species

When aluminum salts affect cellular processes the species that seems most likely to be responsible is Al³⁺. Its high field strength allows it to interact strongly with polar molecules. However, this high field strength also results in the hydration of Al³⁺. At neutral pH, most of the aluminum exists in the form Al(H₂O)₆(OH)₃.⁸ In addition, mono, di, and tetrahydroxylated species also form and dominate a lower and higher pH levels (Fig. 4). In

principle, any of these species could bind to and affect cellular macromolecules.

Upon noticing that at acidic pHs, the aluminum effect on VDAC could be virtually eliminated, it became important to address the question of which species was actually acting on this membrane channel. The effect of pH alone is insufficient because one cannot distinguish between pH effects on the channel and effects on the aluminum species in solution. By examining the ability of other trivalent ions to mimic the action of aluminum one might be able to separate pH effects from effects of altering the level of any given hydrated species.

Gallium and indium are in the same group as aluminum in the periodic table but the levels of their hydroxylated species at a given pH are quite different from that of aluminum (Fig. 4). This is reflected in the amounts of aluminum, gallium or indium which must be added to achieve comparable effects.⁷ At pH 5, 40 μ M of AlCl₃ or InCl₃ are needed to obtain results comparable to those achieved with just 1 μ M

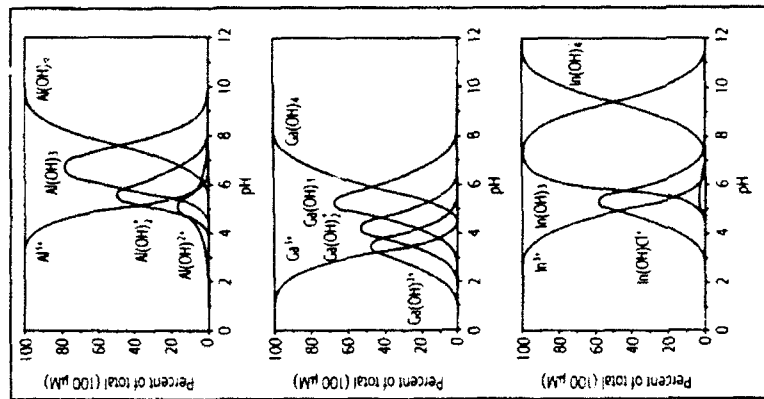


Fig. 4. Distribution of the hydrolysis products of aluminum, gallium, and indium as a function of pH. Formation constants were obtained from Baes & Mesmer⁸ and corrected for ionic strength, osmotic coefficients, and molality of all anions as they described. The concentrations of the species at a particular pH were calculated by solving simultaneously equations for all the known complexes present in solution. The small peak in the indium graph is In(OH)₅. Reproduced with permission from Zhang & Colombini.⁷

GaCl₃ (Fig. 5). By contrast, at pH 7, indium and aluminum were 10 times more potent than gallium. When the potency observed in any experiment was plotted vs. the log of the concentration of each metal species in solu-

tion, the potency was pH and metal independent (Fig. 6). If the free trivalent metal ion were the active species, depending on conditions, comparable effects could be achieved by free metal ion concentrations differing by 8 orders of magnitude! This seems highly unlikely.

A further indication that the free trivalent metal ion is not the active species comes from the observation that La³⁺, an ion that does not form significant amounts of hydrated complexes in solution, is completely ineffective even at concentrations two orders of magnitude greater than those needed to obtain effects with aluminum (Table I).

Other ions (Sc³⁺, Cr³⁺, and Fe³⁺) capable of generating trihydroxide complexes were also able to mimic aluminum's effect on VDAC. The minimum amounts needed to observe effects varied from metal ion to metal ion but, remarkably, correlated very well with regard to the amount of trihydroxide species formed in solution (Table I).

These results strongly support the conclusion that aluminum trihydroxide is the active species. This is surprising not only because it is a neutral species but also because it is very poorly soluble. However, solubility is measured at equilibrium and systems are often away from equilibrium. Most cellular processes are out of equilibrium. The addition of small amounts of an acidic AlCl₃ solution to a solution buffered at neutrality did not result in a visible precipitate as expected at equilibrium. No precipitate was detected days later indicating a stable, supersaturated solution. Such was not the case with CrCl₃, which precipitated in a short time resulting in loss of inhibition of VDAC closure.⁷

Multiple Aluminum Hydroxide Binding Sites on VDAC

If aluminum chloride was added to both sides of a membrane containing VDAC channels, the channels became much less sensitive to the membrane potential. The channels tended to remain in the open state. However, if the aluminum salt were added to just one

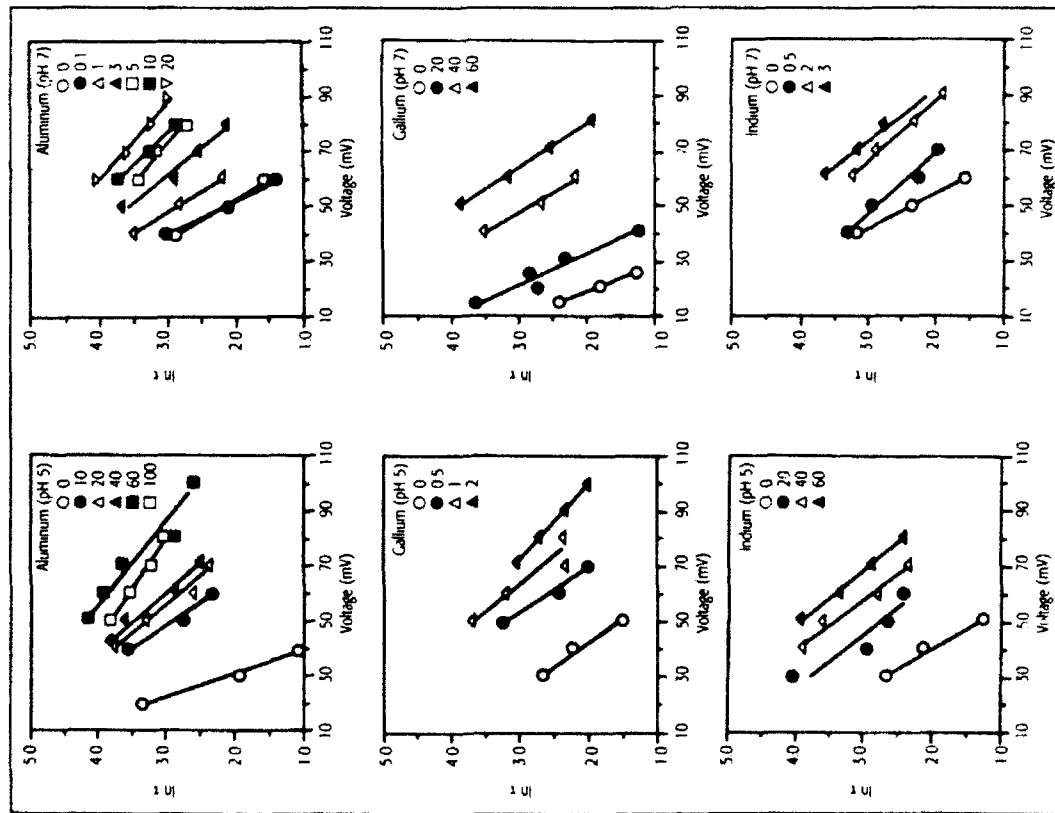


Fig. 5. The pH dependence of the time needed for the current to reach to 1/e of its final value after the application of the indicated potential (for similar records as those shown in Fig. 2). Each panel shows a separate experiment performed under the indicated conditions on a single channel-containing membrane. Sequential additions (to both sides of the membrane) of metal salts were made to final bath concentrations as indicated (in μM). Reproduced with permission from Zhang & Colombini.⁷

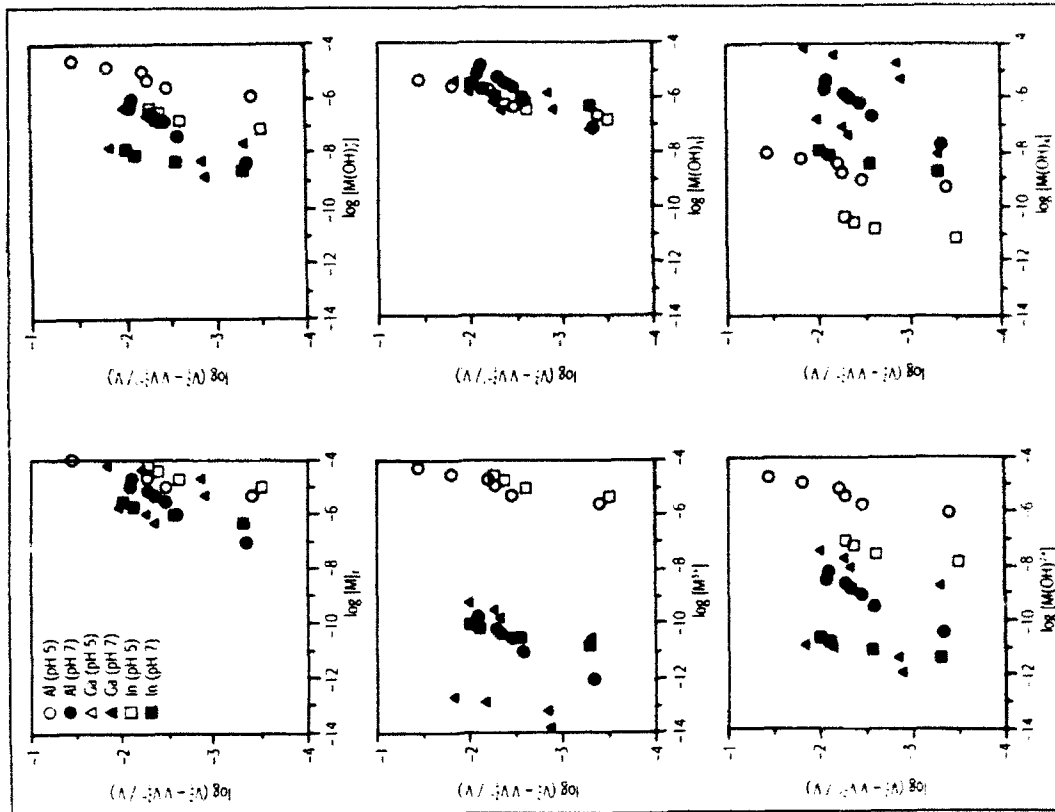


Fig. 6. Correlations between the inhibitory effects on VDAC and the different metal species in solution. Results such as those shown in Fig. 5 were used to construct these panels. The ordinate is the result of a transformation of the data according to a theory⁷ in order to obtain a parameter that represents the effect of aluminum but should vary linearly with the log of the concentration of the species M_1 , is the total metal salt minimum. Reproduced with permission from Zhang & Colombini.⁷

Table 1. Comparison of the characteristics of effective and ineffective metals.

Ion (M ⁺⁺)	Surface Charge Density (e/A ¹)	Ionic Radius (Å)	Lowest Effective or Highest Tested Conc.	
			M(OH) ₂ ^a (M)	Total (M)
Effective				
¹¹ Al ³⁺	0.92	0.51	8 × 10 ⁻⁷	1 × 10 ⁻⁴
²¹ Sc ³⁺	0.45	0.73	7 × 10 ⁻⁷	1 × 10 ⁻⁵
³¹ Ga ³⁺	0.62	0.62	4 × 10 ⁻⁷	5 × 10 ⁻⁶
⁴¹ In ³⁺	0.36	0.81	5 × 10 ⁻⁷	5 × 10 ⁻⁷
⁵¹ Cd ²⁺	0.60	0.63	1 × 10 ⁻⁷	5 × 10 ⁻⁴
⁶¹ Fe ²⁺	0.58	0.64	<4 × 10 ⁻⁴	5 × 10 ⁻⁴
Ineffective				
¹² Mg ²⁺	0.37	0.66	—	5 × 10 ⁻³
²⁰ Ca ²⁺	0.16	0.99	—	5 × 10 ⁻³
²⁷ Co ²⁺	0.31	0.72	3 × 10 ⁻⁶	5 × 10 ⁻⁴
²⁸ Cu ²⁺	0.31	0.72	<6 × 10 ⁻⁴	5 × 10 ⁻⁴
³⁰ Zn ²⁺	0.29	0.74	3 × 10 ⁻⁴	5 × 10 ⁻³
⁵⁵ La ³⁺	0.23	1.02	0 ^a	5 × 10 ⁻⁴

^a Calculated according to Baes and Mesmer⁸ from lowest effective concentration for effective metals and highest concentration tested for ineffective metals at pH 7 (pH 6.6 for chromium), ionic strength = 1.

♦ Essentially not formed at pH 7.

side, the channels would close poorly if the aluminum side were made negative but close very well if the aluminum side were made positive. Indeed, positive potentials applied to the aluminum-containing side resulted in channel closure and poor channel reopening.⁴ This result is illustrated (Fig. 7) with indium as the effector.

This membrane contained a few channels so that individual channel closures were easily visible. The channels closed with positive and negative membrane potentials prior to indium addition. In the presence of 2 μM InCl₃, channels fail to close when the indium-free side was made positive (i.e. negative on the indium-containing side). However, with a negative potential closure was both rapid and extensive. A return to -10 mV, a potential which al-

lowed the channels to reopen prior to indium addition, resulted in a lower current (compare levels at two adjacent arrows) indicating that most of the channels remained closed. The reapplication of a positive potential also resulted in a smaller current because some channels were still closed. Positive potentials usually induce the channels to reopen if these are sufficiently large and applied for long enough time.

The asymmetric behaviour resulting from asymmetric aluminum addition can be understood in terms of two aluminum binding sites able to translocate through the membrane (Fig. 8). Aluminum added to one side binds to a site that would move to the other side if a negative potential were applied to the aluminum-containing side. The binding of aluminum prevents this and so the channels do not

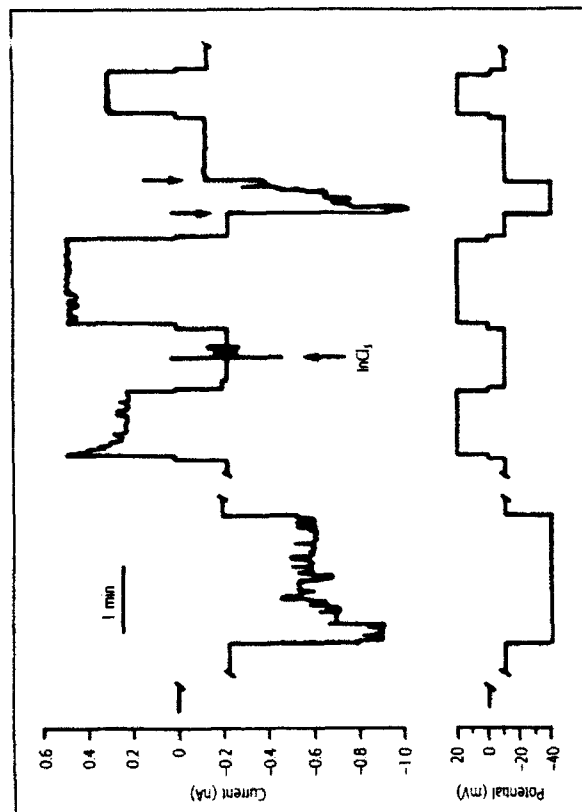


Fig. 7. Asymmetric addition of InCl₃ resulted in inhibition of one gating process and enhancement of the other. Experimental conditions were similar to those in Fig. 2. InCl₃ was added to one side of the membrane as indicated to a final conc. of 2 μM. The sign of the applied potential refers to the side of the membrane lacking indium. Reproduced with permission from Zhang & Colombini.⁵

close. However, the channels do close if a positive potential is applied because another domain is moving from the opposite side to the aluminum-containing side. Once there, aluminum binds inhibiting the re-opening of the channel. While other explanations may account for the observations, this is the most straight-forward interpretation.

Conclusions

Although investigations on the effects of aluminum salts on membrane channels are at an embryonic stage, it is already clear that the effects observed to date are among the most potent effects of aluminum so far described. Aluminum opens the channels or keeps them open presumably by inhibiting the gating proc-

esses that regulate these channels. At least in the case of VDAC channels from mitochondria, the effects are mediated by a neutral species (aluminum trihydroxide) most prevalent at physiological pH. Only time will tell how widespread the phenomenon is and whether aluminum hydroxide turns out to be the active species in other aluminum-influenced cellular processes.

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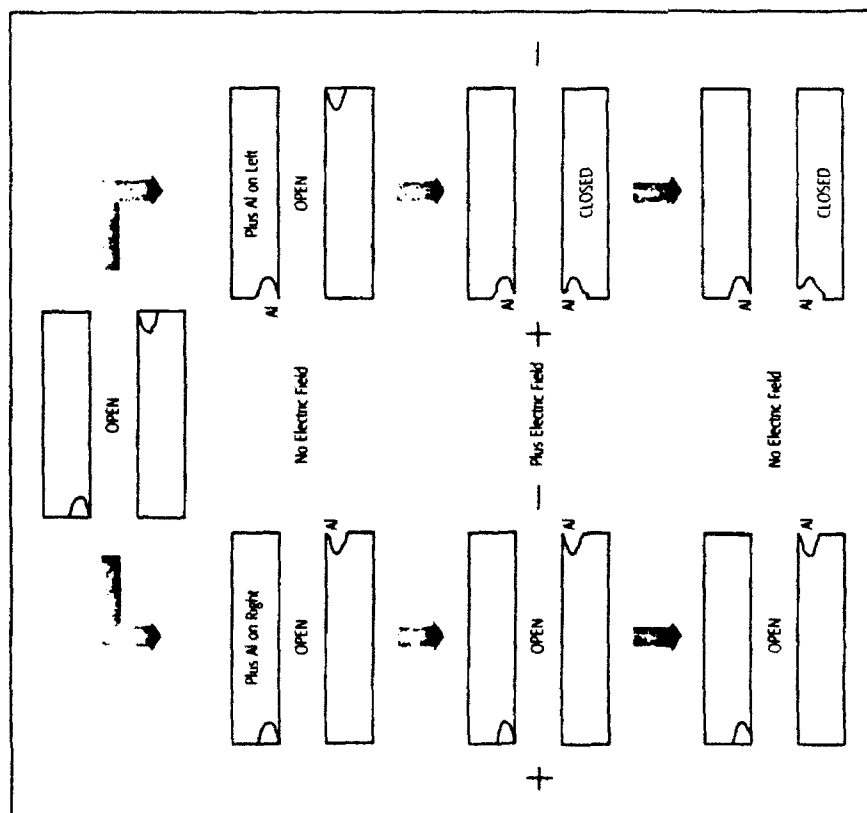


Fig. 8. Schematic drawing of a model that accounts for the asymmetric effect of aluminum. The channel is depicted in longitudinal cross-section with the shaded rectangles being the walls of the channel. The small notches on the channel represent the binding sites for aluminum. Starting at the top with an open channel, aluminum salt is added either to the right or left of the channel. This results in no effect until a membrane potential is applied. If the aluminum-containing side is made negative, the channel fails to close but closure occurs if it is made positive. When the potential is removed, the closed channel does not reopen because aluminum is bound to the site that translocated through the membrane as a result of channel closure. Reproduced with permission from Zhang & Colombini.⁸

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