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The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion

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A synthetic polyanion has been found to modulate the properties of the mitochondrial outer membrane channel, VDAC. This 10 kDa polyanion, first synthesized and described by Konig and co-workers, is a 1:2:3 copolymer of methacrylate, maleate, and styrene. It had been shown to interfere with the access of metabolites to the mitochondrial inner spaces. Here we show that, at nanomolar levels, the polyanion increases the voltage dependence of VDAC channels over 5-fold. Some channels seem to be totally blocked while others display the higher voltage dependence and are able to close at very low membrane potentials (5 mV). At 27 μg/ml polyanion, VDAC channels are closed while inserted into liposomes in the absence of any applied potential. The closed state of VDAC induced by the polyanion has similar properties to the closed state induced by elevated membrane potentials. The physical size of the polyanion-induced closed state (in VDAC-containing liposomes) is about 0.9 nm in radius. How this estimate fits with estimates of the channel's open state and estimated volume changes between the open and closed states, is discussed.

Introduction

All substrates used by the mitochondrion and all products which it exports to the cytoplasm must cross the outer mitochondrial membrane. The importance of this membrane in regulating this traffic is not clear although there are indications that such regulation may occur [1]. Substances that modify the permeability of the outer membrane are of interest because they may be used as tools to (1) study the function of the outer membrane, (2) assess the role that this permeability barrier plays in overall mitochondrial function, and (3) gain insight into the properties of the major permeability pathway of the outer membrane, the channel-forming protein, VDAC [2–6].

König and co-workers have reported [7,8] that a synthetic polyanion (a copolymer of methacrylate, maleate, and styrene in 1:2:3 ratio with average molecular weight of 10000) inhibited a variety of mitochondrial activities. These include the exchange of intramitochondrial malate for either citrate, oxoglutarate, or phosphate, the uptake of ATP or ADP, and ATPase activity. These effects occurred at similar doses of polyanion (the reported K_i values are 1 to 4 μM). It is possible that the similar doses indicate a common mode of action. However, at least one of the effects (ATPase activity) appears to be the result of direct interaction between the polyanion and the protein performing the function being tested. Nevertheless blockage of the outer membrane channel, VDAC, would interfere with all the functions and would

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do so at the same dose of polyanion (small variations in the K_i could result from interference of the anions being studied with polyanion binding). The large size of the polyanion makes it unlikely to cross the outer membrane through VDAC (reported molecular weight cut-offs for VDAC are 4000 to 6000 [3,9]).

Although ultrastructural evidence for channels in the mitochondrial outer membrane was first reported in 1965 [10], the existence of these channels was not demonstrated until 1976 when they were isolated from *Paramecium* mitochondria and named VDAC [11]. These channels are highly conductive (4.5 nS in 1 M KCl, 5 mM $CaCl_2$ [12]) and permeable to large molecules [3,9] in the absence of a transmembrane potential (open state) but their conductance drops when a voltage-induced conformational change converts the channels to lower-conducting (closed) states [11,13] (approx. 20 mV are needed). This reduction in channel permeability at relatively small transmembrane voltages results in reduced permeation of substrates such as succinate (10-fold reduction; Colombini, M., unpublished observation). In this paper we report that small doses of this polyanion drastically alter the permeability properties of VDAC inducing these channels to enter less conducting (closed) states.

Methods

Isolation of VDAC channels

All experiments were performed on VDAC channels isolated from *Neurospora crassa* mitochondria. The wall-less mutant used for these studies was obtained from the American Type Culture (No. 32360). The cells grown and maintained as previously described [16]. Some experiments were performed with VDAC isolated from whole mitochondrial membranes (isolated as in Ref. 12) while others used purified outer membranes (isolated as in Ref. 19). The outer membranes or the whole mitochondrial membranes were stored at either $-20\,^{\circ}C$ or $-80\,^{\circ}C$ in 1 mM KCl, 1 mM Tris-HCl (pH 7.5) supplemented with dimethylsulfoxide (DMSO) at 15% (v/v). For the planar membrane experiments, 5 μl of a 5% Triton X-100 solution were added to 20 μl of membrane suspension (freshly thawed out with DMSO present) and the resulting solution was kept at room temperature until it was used (within a few hours).

Planar phospholipid membrane studies

The planar phospholipid membranes were generated by the monolayer technique of Montal and Mueller [14] and essentially as previously described [11]. Soybean phospholipids (purified by organic solvent extraction [15]) were used to make these membranes (in other lipids more complex results may be obtained, see Results). Unless otherwise specified, all experiments presented were performed in unbuffered 1.0 M NaCl, 5 mM $CaCl_2$ (KCl should not be used, see Results). Permeability changes in the membrane were measured by recording current under voltage-clamp conditions as previously described [11]. The side to which the VDAC-containing solution was added was defined as the cis side. Normally channels were inserted into the membrane by adding 5 to 10 μl of the 1% Triton X-100 extract of mitochondrial membranes to 4 to 6 ml of aqueous solution on the cis side of the membrane.

Liposome studies

VDAC-containing liposomes were generated essentially as described previously [12]. The ability of non-electrolytes to permeate the liposomes through VDAC was determined as previously reported [12] except for the indicated changes. The liposomes were generated in 8 mM NaCl, 3 mM Na_2EDTA (pH 7.0) at a lipid concentration of 13 mg/ml. 20 μl of this suspension were diluted into 1.0 ml of 220 mM NaCl, 3 mM Na_2EDTA (pH 7.0) (experiments were also performed at lower NaCl concentrations in order to establish the importance of ionic strength). Polyanion was added to the final concentration indicated in the figure legends. When the liposomes had stabilized (constant absorbance level), they were induced to shrink by the addition of a non-electrolyte to the medium. This shrinkage was monitored as an increase in turbidity at 400 nm. The shrinkage was followed by reswelling if the non-electrolyte was able to permeate the liposomes (a turbidity decrease). The maximal rate of this reswelling was taken as a measure of the ability of the non-electrolyte to permeate through VDAC.

Polyanion solutions

The polyanion (methacrylate/maleate/styrene, 1 : 2 : 3) was prepared by one of us (T.K.; see Ref. 7 for details of synthesis and characterization) and neutralized with NaOH to produce the stock solutions. For the planar membrane experiments, a 1 mg/ml stock solution was made with final pH of 7.2. For the liposome experiments, a 15 mg/ml stock solution was made with a final pH of 7.5. The 1 mg/ml stock was clear while the 15 mg/ml was slightly cloudy. Several dilutions of the stock were made to serve as working solutions. These latter solutions were clear. All solutions were stored at 4 °C.

Results and Discussion

Two sets of experiments are presented. In the first set, planar phospholipid membranes containing VDAC channels were used to study the ability of the polyanion to change the properties of these channels. The ability of small ions to flow through the channels was monitored by measuring the current flow through the membrane. In the second set, the polyanion was added to liposomes containing VDAC channels. The ability of large non-electrolytes to penetrate the liposomes through VDAC was determined by measuring volume changes in the liposomes.

Planar membrane experiments

Planar phospholipid membranes were made as described in Methods using a soybean phospholipid mixture. Triton X-100 solubilized VDAC channels were inserted into the planar membranes as previously described [2] by adding a 5 to 10 μl aliquot to the aqueous solution on one side (defined as the cis side) of the membrane. After the channels had spontaneously inserted into the membrane, their voltage dependence was examined by applying an elevated potential. The upper left trace in Fig. 1 shows voltage-induced channel closure with application of 41 mV to the trans side of the membrane. The current instantaneously increased as the voltage drove ions through the channels and then decayed as channels closed. Shortly after this test, the current amplification was increased 2.5-fold and the trace on the rest of the figure was recorded. The applied voltage was 5.1 mV. At this voltage VDAC channels do not normally close (notice, at the extreme left end of the figure, that 10 mV did not result in channel

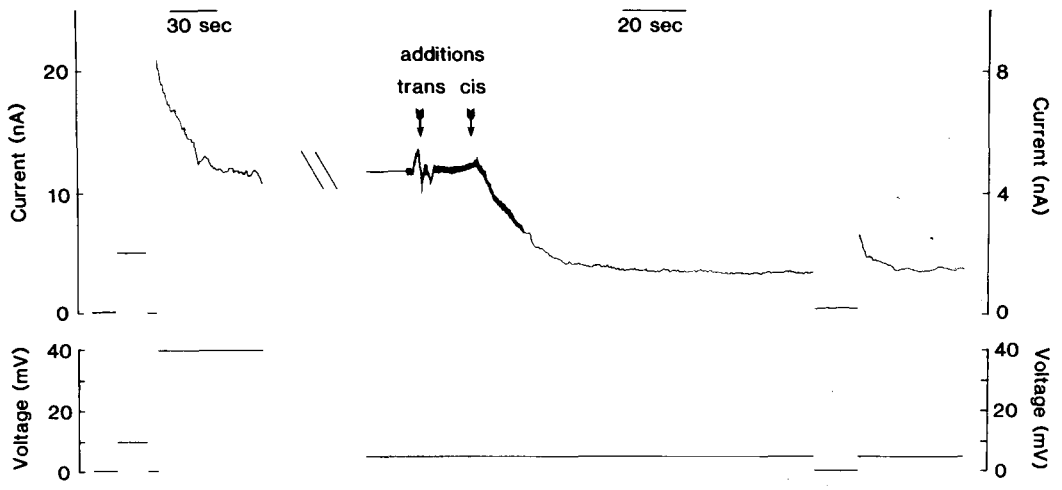

Fig. 1. Inhibition of VDAC conductance by the polyanion. The upper tracings are of the current records in response to the applied voltages shown below. The records on the left side were obtained at a 2.5-fold lower gain as compared to the records on the right. The time scale was also changed and this is indicated by the calibration bars at the very top. At the points indicated by the arrows, 200 μl of 125 μg/ml polyanion was added to cis and trans sides of the membrane (final concn. = 5 μg/ml). The noisy trace around the points of addition is a stirring artifact. The membrane potentials in this figure refer to the trans side (the cis side was equated to zero).

closure). The applied voltage allowed us to follow changes in channel conductance resulting from the addition of the polyanion.

At the points indicated by the arrows, polyanion (5 μg/ml final concn.) was added to the cis and trans sides of the membrane (the stirring generated the electrical noise recorded as a darker trace). The resulting decrease in current appears to have two components: (1) increase in voltage dependence so that channels close at lower potentials, (2) loss of conductance, perhaps due to blockage of some channels. These are indicated by the test on the right of the figure. Following the decay in current which occurred with an applied potential of 5.1 mV, the potential was reduced to 0.2 mV and returned to 5.1 mV. The short time at 0.2 mV allowed the channels that had closed to reopen. That the current did not return to the value obtained prior to polyanion addition, was not due to insufficient time at 0.2 mV (channel opening is a fast process [2,11] and was complete prior to the reapplication of the membrane potential). Rather, all the channels that could open, did open. The lost conductance may represent channels that were blocked or unable to open. The fraction of conductance that is lost varies from experiment to experiment but is clearly related to the amount of polyanion added. The channels, that did open, closed once again at 5.1 mV, a potential at which these channels do not normally close. The rapid rate of closure seen here at 5.1 mV would have required an applied potential of at least 40 mV in the absence of the polyanion.

The experiment shown in Fig. 1 indicates that one of the actions of the polyanion is to increase VDAC's voltage dependence. By measuring the conductance of a VDAC-containing membrane as a function of applied potential, it is possible to obtain a detailed description of VDAC's voltage-dependent behavior. The plots shown in Fig. 2 were obtained in the absence and in the presence of 3 μg/ml polyanion (the same amount of polyanion was added to each side of the membrane). The voltage was applied in the form of a triangular voltage wave and the measured current was used to calculate the membrane conductance. The results were normalized by dividing the conductance values for each curve by the observed conductance at zero applied potential. The striking

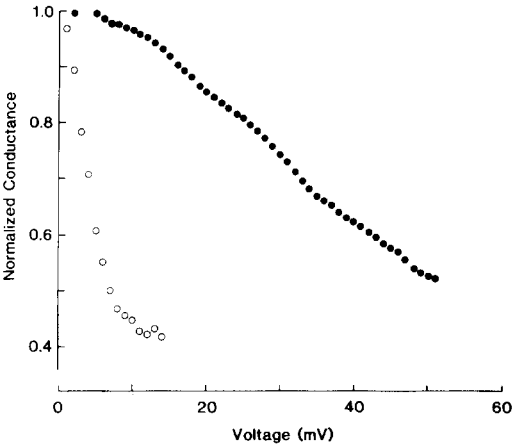

Fig. 2. The polyanion increased the steepness of the voltage dependence. The figure shows the steady-state conductance of control (filled circles) and polyanion-treated (3 μg/ml, open circles) channels as a function of applied potential. The current flowing through the membrane was measured while the voltage was varied at 37.6 mV/min. The current measurements made while the voltage was decreasing were the only ones that were used. The conductance was calculated for each point and then normalized to the maximal conductance.

increase in voltage dependence induced by the polyanion is demonstrated by the rapid drop in conductance with increase in applied potential. The steepness of the voltage dependence increased over 5-fold (estimated as in Ref. 11).

Under the experimental conditions used to generate the results shown in Figs. 1 and 2, the polyanion alone had no detectable effects on planar membranes to which no VDAC had been added. However, at high polyanion concentrations (15 μg/ml) and with KCl as the major salt, the membrane conductance increased with addition of polyanion (no VDAC channels). The conductance increase was noisy, slow, and sustained, until (after some time) the membrane broke. This was not observed in NaCl medium, at 15 μg/ml polyanion concentration, unless the phospholipid used to generate the membrane was diphytanoylphosphatidylcholine. The ability of this polyanion to increase the permeability of phospholipid membranes was also observed in the liposome experiments at 0.25 mg/ml polyanion concentration using NaCl as the major salt (see below). The mechanism of this permeability increase is unclear.

Liposome experiments

The planar membrane experiments indicated that the polyanion might block VDAC channels. However, in those experiments, what was measured was a current due to the flow of small ions through the channels. The reduction of this flow by the polyanion could have been due to a physical blockage, an electrostatic effect or perhaps an induction of a conformational change in VDAC converting it from the open to the closed state. If the polyanion were to bind to the channel's mouth, its highly charged nature might allow cations to pass through anyway but at a reduced rate. On the other hand, large molecules should be impermeable. An electrostatic effect would only interfere with ion movement, non-electrolytes should not be effected. If a conformational change in VDAC were to take place, the size of the molecules that could permeate the channels should be reduced. In order to distinguish between these possibilities, experiments were performed with measured the flux of non-electrolytes into VDAC-containing liposomes.

VDAC-containing liposomes were preincubated (for at least 2 h) in 220 mM Na_2EDTA (pH 7.0) at room temperature, in the absence or with varying concentrations of polyanion. After liposome size, as measured by turbidity at 400 nm, had stabilized, a non-electrolyte was added in order to apply an osmotic pressure gradient. Fig. 3 shows a sample of the results obtained using polyethylene glycol (PEG) 1500. The PEG caused the liposomes to shrink (as evidenced by the increase in absorbance) but whether this was followed by reswelling depended on the dose of polyanion added. At 27 μg/ml, no detectable reswelling was observed while with lower and higher concentrations, reswelling occurred. As previously reported [12], PEG 1500 can permeate through VDAC so that the reswelling observed in the absence of polyanion can be interpreted as PEG 1500 entering the liposome and being followed by water. Higher concentrations of polyanion make the liposomes progressively less permeable to PEG 1500, perhaps by closing VDAC channels. At very high polyanion concentrations, the polyanion seems to allow PEG 1500 to enter the liposomes by another pathway. This is consistent with the increased permeability observed when a high polyanion concentration was applied to planar membranes (see above).

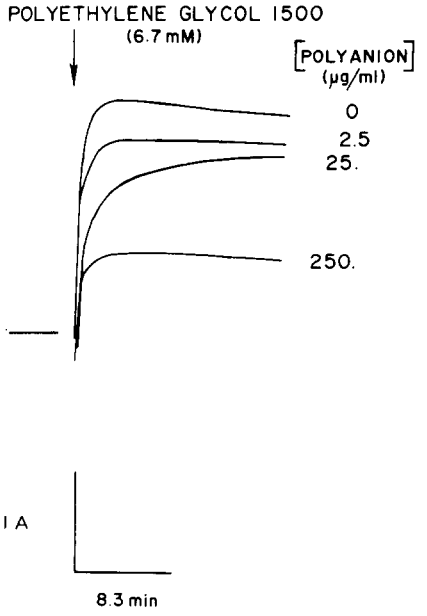

Fig. 3. The polyanion alters the turbidity changes induced by the addition of polyethylene glycol (mol.wt. 1500) to VDAC-containing liposome. Absorbance measurements were made at 400 nm. The liposomes were allowed to equilibrate with or without the polyanion for at least 30 min. At the point indicated, 80 μl of 100 mM polyethylene glycol(PEG) 1500 were added to 1.0 ml of liposome suspension. The four traces were superimposed in such a way that the PEG additions coincide and the absorption levels prior to PEG addition, overlap (these levels were quite close anyway).

The results of many experiments with four different non-electrolytes are summarized in Fig. 4. The maximal observed rate of absorbance decrease (as a measure of the maximal rate of reswelling) was measured in all cases and expressed as a fraction of the rate observed in the absence of polyanion. This normalization helped to factor out day-to-day variation in the liposome behavior so that means and standard errors could be presented. The non-electrolytes used ranged in size as follows: inulin (M_r = 3000–5000), PEG 1500 (M_r = 1300–1600), gamma-cyclodextrin (M_r = 1297), and stachyose (M_r = 666.6).

The ability of the polyanion (at 27 μg/ml) to inhibit non-electrolyte permeation depended on the size of the non-electrolyte. The large ones (inulin and PEG 1500) were profoundly inhibited while stachyose was not significantly affected. The

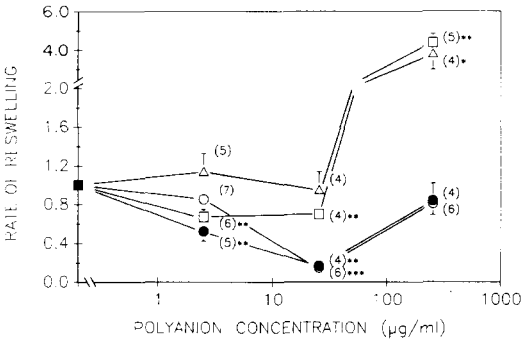

Fig. 4. Normalized reswelling rates induced by different non-electrolytes (○, inulin; ●, PEG 1500; □, gamma-cyclodextrin; △, stachyose), as a function of polyanion concentration. Experiments such a those shown in Fig. 3, were performed and the maximal rates of absorption decrease (as a measure of liposome reswelling) were determined by measuring, whenever possible, the slope at the inflection point. The data was normalized by dividing each value by the slope in the absence of polyanion (this was done to reduce day-to-day variability and obtain the standard errors shown). The numbers next to the standard error bars are the number of estimates. *T* tests were performed to assess the significance of the differences between experiments done in the presence and absence of the polyanion. The levels of significance are given by: * = < 0.05; ** = < 0.01; *** = < 0.001.

inhibition of gamma-cyclodextrin permeation was significant but not as great as that observed with the higher molecular weight compounds. Indeed, at 27 μg/ml polyanion, the reswelling rates for PEG 1500 and insulin treatments were not significantly different from zero (at 95% and 99.9% confidence levels, respectively) while the cyclodextrin treatments were significantly different from zero (at the 99.9% level). Thus the permeability cut-off for the polyanion-treated channel is just about at gamma-cyclodextrin.

At 270 μg/ml polyanion, the liposomes behaved as if they were permeable to all the probe molecules. The polyanion seems to induce non-specific permeability pathways in the liposomes.

VDAC's closed state is permeable to ions but at a lower level than the open state indicating that the closed state has a smaller pore size. If the polyanion were inducing VDAC channels to close, the closed state could allow the smaller non-electrolytes to pass while blocking passage to the larger molecules. The experiments are consistent with this hypothesis and are not consistent with a blocking hypothesis that would predict all but the smallest ions to be impermeable. If the polyanion blocked the channel, the small ions might permeate through the negatively charged mesh formed by the polyanion but the non-electrolytes used in this study should not be able to penetrate (note that the Stokes-Einstein radius for stachyose is about 7Å[17]). The third possibility mentioned above was that the polyanion might interfere with ion flow through VDAC by way of an electrostatic effect. Since profound effects were observed on non-electrolyte permeation, this hypothesis is also not tenable.

The higher doses of polyanion needed to observe effects in the liposome experiments were probably the result of two factors: (1) no voltage

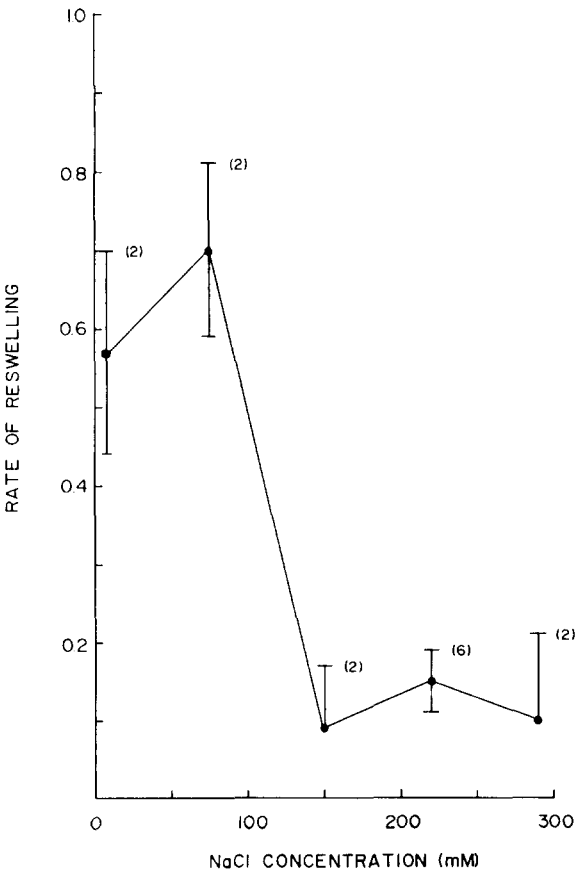

Fig. 5. Salt dependence of the polyanion inhibitory effect. Similar experiments, to those shown in Fig. 3, were performed. The reswelling rates were normalized to the rates observed in the absence of polyanion. PEG 1500 was used to apply the osmotic pressure and all experiments (whose results are shown) were performed in the presence of 27 μg/ml polyanion. The day-to-day variation is shown by the standard error bars. The number next to each bar is the number of trials.

could be applied across the membranes in the liposome experiments so, most likely, higher doses were required to close the channels in the absence of an electric field; (2) lower salt concentrations were used in the liposome experiments which means that the effective concentration of polyanion next to the membrane was probably lower due to less salt screening of the membrane surface potential. The importance of the latter effect was indicated by a salt dependence of the liposome reswelling rates (Fig. 5). A marked reduction in the reswelling rate, and thus liposome permeability to PEG 1500, occurred with increasing salt concentration and therefore we chose to use 220 mM NaCl for the results reported in Figs. 3 and 4.

Taken together, the results of the two different experimental approaches demonstrate that nanomolar quantities of this synthetic polyanion influence the properties of the mitochondrial channel, VDAC. The polyanion causes the channel to be less permeable, probably by causing the channel to be converted to its less permeable, closed, conformation. This 'closed' state, generated, by the polyanion has a molecular weight cut-off about the size of gamma-cyclodextrin (i.e. 1297). Gamma-cyclodextrin is a doughnut-shaped molecule of radius 0.84 nm [18]. Since the cyclodextrin still seems to penetrate, a reasonable estimate for the radius of this closed state is 0.9 nm. Estimates for the radius of the open channel range from 0.9 to 2 nm [13,21,22] *. The low estimate is clearly unreasonable. The other estimates *, 1.25 and 2 nm would indicate a change in the channel's cross-sectional area upon closure (by polyanion) of 48 and 80%, respectively. Ignoring changes in the electrostatic barriers, this is consistent with an observed conductance decrease of 60% (Ref. 13).

Estimates have been reported [23] for changes in the volume of VDAC's pore upon voltage-dependent closure of 20–40 nm^3. Using open channel radii of 1.25 and 2.0 nm, the volume changes (assuming cylindrical channels of length 5.4 nm [22]), resulting from polyanion-induced closure, are 12.8 and 54.1 nm^3. Thus, considering the uncertainties in all of the estimates, all results are consistent with the polyanion inducing VDAC channels to close to the same closed state achieved by applying an electric field.

It is unclear, at present, how specific these findings are to the particular composition of the polyanion. Indeed, the synthetic nature of the substance means that it consists of a mixture of compounds and some of these are probably more effective than others. This polyanion or similar molecules may prove to be useful probes of the functions of VDAC, e.g. does VDAC serve as a conduit for proteins through the outer membrane?

Similar effects, to those reported here can be obtained using other polyanions (e.g. dextran sulfate, 8 kDa) but higher doses are needed to obtain comparable results [20]. We are exploring the possibility that a natural agent may be present in cells that acts like the polyanion in regulating VDAC closure. Such an agent could regulate the molecular traffic across the mitochondrial outer membrane and perhaps regulate the rate of cellular energy production.

* The physical size of the aqueous pore formed by the VDAC channel has been estimated, by different methods, to be anywhere from 0.9 to 2.0 nm [13,21,22] in radius. This variability stems from the various methods used to arrive at the estimate. The simplest and most naive approach is to calculate the radius of the pore from the single-channel conductance assuming that the specific conductance within the channel is the same as that in the bulk phase (ignores effects of electric fields within the channel). This calculation yields the lowest estimate (0.9 nm [21]). Even after the calculation is performed correctly considering the access resistance [24], the value is only (1.0 nm). By taking advantage of the ordered arrays of VDAC channels in the outer membrane of *N. crassa* mitochondria, computer filtration of electron micrographs of negatively stained membranes yield a pore size of 1.25 nm [22]. By determining the largest non-electrolyte that can cross a membrane through VDAC, an estimate of 2.0 nm was obtained [13]. The latter may be an overestimate due to the flexibility of the large polymers and the estimate from negatively stained membranes may be an underestimate due to the packing properties of the particles of stain.

Acknowledgement

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