

VDAC Channels Differentiate between Natural Metabolites and Synthetic Molecules

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Abstract. VDAC provides the major permeability pathway through the mitochondrial outer membrane by forming voltage-gated channels with pore radius of 1.2–1.5 nm. We find that VDAC can select among comparably-charged molecules with a much smaller effective radius, 0.4–0.5 nm. The molecules studied were the nucleotides, ATP, UTP, NADH and synthetic anions, tetraglutamate (T-Glu) and 1-hydroxypyrene-3,6,8-trisulfonate (HPTS). VDAC channels were reconstituted into planar phospholipid membranes bathed in 1.0 M NaCl (buffered to pH 8.0). The nucleotides decreased the conductance of VDAC for NaCl demonstrating that they could permeate into the channel. In contrast, T-Glu and HPTS did not change the single-channel conductance, indicating exclusion from the channel. Reversal potential measurements report near ideal selectivity of Na⁺ over T-Glu. The nucleotides increased single-channel noise as they penetrated into the channel, while T-Glu had no effect. HPTS increased noise, but unlike NADH, this was not voltage-dependent when HPTS was added asymmetrically, indicating no penetration into the channel. The differences in effective size and charge cannot explain the difference in permeation characteristics. Thus VDAC must select among these based on shape and charge distribution. We propose that the electrostatic environment within the channel has been evolutionarily selected to favor the passage of adenine nucleotides.

Key words: Mitochondria — Planar membranes — Permeation — Large channel — Current noise — ATP

Introduction

Our understanding of the process by which ions and molecules permeate through membrane channels is still rather limited. This is especially true for large transmembrane channels, which are traditionally regarded as simple “molecular sieves” and assumed to select solutes solely based on their size and charge. High selectivity has traditionally been associated with membrane transporters that function as carriers (Bowman & Levitt, 1977). Narrow-pore channels, such as Na⁺ and K⁺ channels from the excitable cells, are also known to be highly selective, but proteins that form large aqueous pores are presumed to allow passage without sufficient interaction with the protein walls to provide selectivity above general electrostatic effects. However, the reason for the existence of channels with large aqueous pores is to allow the passage of large molecules. As these molecules pass through the pore they may indeed interact with the walls in a way that allows for specificity.

Specificity in the passage of metabolites through channels in membranes forming intracellular compartments would clearly be of importance. Intracellular membranes compartmentalize different cellular regions, providing specialized environments for specialized functions. Rapid exchange of specific metabolites between different compartments is sometimes essential to survival and could be achieved by channels specialized for that function. Exchange of adenine nucleotides between the mitochondrial spaces and the cytosol is at times limited by the permeability of the outer mitochondrial membrane (Gellerich & Kunz, 1987; Gellerich, Khuchua & Kuznetsov, 1993). The VDAC channels that perform this function (Benz et al., 1988; Liu & Colombini, 1992; Gellerich et al., 1993; Lee, Xu & Colombini, 1996) may therefore have been under strong selection pressure to maximize their ability to translocate

adenine nucleotides without allowing small proteins such as cytochrome *c* to leak out. This adaptation may have tuned the VDAC channel to such an extent that molecules of size and charge similar to ATP may not permeate as well because their shape and charge distribution may not match the structure of the channel.

The VDAC channel is believed to be responsible for most of the metabolite flux across the mitochondrial outer membrane (Colombini, 1979; Benz et al., 1988; Liu & Colombini, 1992; Lee, Zizi & Colombini, 1994; Vander Heiden et al., 2001). When reconstituted into planar phospholipid membranes, VDAC forms large aqueous pores (Colombini, 1994) that are open at low applied voltages (<20 mV) and exhibit weak anion selectivity in the open state for small ions (4:1 Cl⁻:K⁺, measured in a 0.06/0.12 activity gradient). A working model of the VDAC pore (Song et al., 1998*a*; Mannella, Guo & Cognon, 1989; Colombini, Blachly-Dyson & Forte, 1996) envisages a barrel with an internal diameter of 2.4–3.0 nm composed of a tilted (approximately 45°; Abrecht et al., 2000) α -helix and thirteen β -strands (Song et al., 1998*b*). Experimentally, it forms an aqueous pore that allows uncharged polymers such as inulin, dextran and polyethylene glycol of molecular weight about 5000 to cross membranes (Colombini, 1980; Zalman, Nikado & Kagawa, 1980). However, the apparent size of the channel is very different for non-electrolytes and for charged molecules. Anions, such as ATP, are favored due to the net positive charge within the channel. However, the specialization goes beyond net charge.

By using current noise analysis, we have found that VDAC channels can distinguish between ATP and UTP (Rostovtseva et al., 2002). The evidence favors the existence of a binding site inside the VDAC channel that discriminates between *purines* and *pyrimidines*. The nucleotide-generated low-frequency noise obeys the following sequence: β -NADPH > β -NADH = α -NADH > ATP > ADP > β -NAD \geq AMP > UTP. The ability to generate current noise spans a forty-fold range for different nucleotides from barely measurable to highly pronounced. In contrast to this wide range, addition of any of these nucleotides reduces the single conductance for small ions to a very similar extent, indicating a similar residency time inside the channel. Thus, the selectivity among nucleotides observed based on noise generation likely arises from a process other than ability to penetrate into the channel.

In order to determine whether VDAC channels can select among molecules of similar size and charge, we extended our study to non-physiological molecules, tetraglutamate (T-Glu) and 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS). These substances were chosen as non-physiological analogues of ATP because of their similar size and charge as ATP. We

will present evidence that these molecules are excluded from VDAC channels. Thus, the ability of the charged molecules to permeate through large channels may depend on their shape and the distribution of charge over their surface rather than simply their size and net charge.

Materials and Methods

CHEMICALS

ATP as disodium salt, UTP as trisodium salt, and 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS) as trisodium salt were purchased from Sigma Chemical (St. Louis, MO). 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (known as BAPTA) was purchased from Molecular Probes (Eugene, OR) as tetrasodium salt (*see* Fig. 1 for structures).

A tetramer of glutamic acid was synthesized by Commonwealth Biotechnologies (Richmond, VA) as the trifluoroacetic (TFA) salt. Tetraglutamate (T-Glu) was purified from TFA by replacing TFA with acetic acid. The tetraglutamate-TFA powder was mixed 3 times with glacial acetic acid followed by centrifugation. The remaining acetic acid was washed out with water by multiple drying using a rotary evaporator. Purified T-Glu was titrated to pH 8.0 by using a stoichiometric amount NaOH (4:1 on a molar basis).

NOISE AND SINGLE-CHANNEL CONDUCTANCE MEASUREMENTS

VDAC channels were isolated from *Neurospora crassa* mitochondrial outer membranes and purified according to standard methods (Mannella, 1982; Freitag, Benz & Neupert, 1983). Bilayer membranes were formed from monolayers made from a 1% solution of diphtanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in hexane (Aldrich Chemical, Milwaukee, WI) on a 70–90 μ m diameter aperture in a 15- μ m thick Teflon partition that separated two chambers (modified Montal and Mueller technique, 1972). The total capacitance was 70–80 pF and the film capacitance was 30–35 pF. Aqueous solutions of 1.0 M NaCl and 1 mM CaCl₂ were buffered with 5 mM HEPES at pH 8.0–8.2. All measurements were made at room temperature.

Single-channel insertion was achieved by adding 0.1–0.3 μ l of a 1% Triton solution of purified VDAC to the 2.0 ml aqueous phase in the “*cis*” compartment while stirring. After a single channel was inserted and its parameters were recorded, membrane-bathing solutions in both compartments were replaced by solutions containing ATP, UTP, T-Glu or other compounds. In this way the effects of nucleotides were observed on the same channel. At the end of each experiment the contents of both compartments were taken to measure conductivity using a CDM 80 conductivity meter (Radiometer, Copenhagen, Denmark) in order to verify the effectiveness of the perfusion. The concentrations of HPTS and BAPTA were measured by their absorbance at 455 nm and 278 nm, correspondingly.

Solutions containing the test compound were produced in such a way as to maintain the molality of the NaCl. Thus, 1.0 M NaCl solutions were used to dissolve the test compounds and while the molarity changed the molality did not. In this way, if the test compound did not penetrate the channel, the solution of NaCl in the channel would be the same as if the channel had been exposed to the same NaCl concentration without the test compound. Decreases in the conductance of the channel in the presence of the test compound would be due to penetration of the test compound into the lumen of the channel.

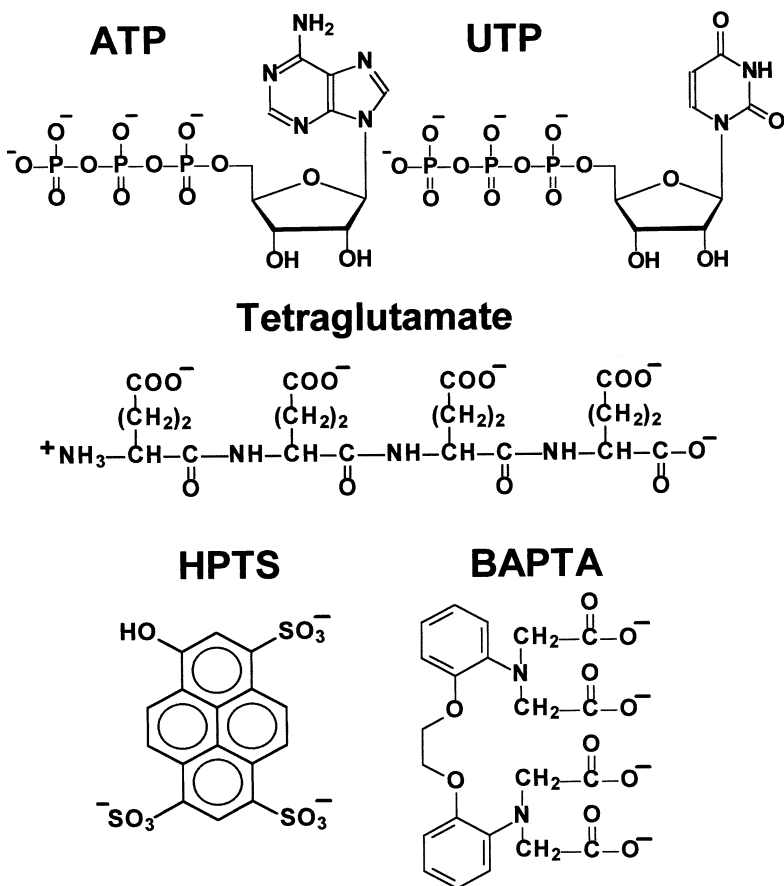


Fig. 1. Chemical structures of probe molecules used: ATP, UTP, tetraglutamate, HPTS and BAPTA.

Addition of the test compound to 1.0 M NaCl solutions sometimes required some neutralization with NaOH to achieve pH 8.0. This dilution of the sodium chloride solution, while small, influenced the results significantly, especially conductance measurements. Thus, conductance measurements made of the bulk solution and on channels were compared to control sodium chloride solutions diluted to the same extent with water. Only relative conductances are reported, because we are interested in how the nucleotides change the conductance of the medium. This dilution also affected current-noise measurements. For equilibrium sources of conductance fluctuations the amplitude of the current noise-power spectrum is proportional to the square of the current (DeFelice, 1981; Kasianowicz & Bezrukov, 1995). Thus, all values were adjusted to compensate for current changes arising from the dilution and expressed at the noise level expected if no dilution had occurred. This correction was insignificant except at low noise levels.

The membrane potential was maintained using Ag/AgCl electrodes with 3.0 M KCl, 15% agarose bridges assembled within standard 200- μ l pipette tips (Bezrukov & Vodyanoy, 1993). The potential is defined as positive when it is greater at the side of protein addition (*cis*). The noise analysis of a single VDAC channel was done as described earlier (Rostovtseva & Bezrukov, 1998; Rostovtseva et al., 2002). Amplitude and power spectrum analysis was done using software developed in-house.

MEASUREMENT OF THE REVERSAL POTENTIALS

The reversal or zero-current potential was measured to assess the selectivity in single or multi-channel membranes. The reversal po-

tential of an ideally cation-selective membrane was found by measuring the reversal potential of gramicidin-doped membranes under identical conditions. Since the same electrodes and the same T-Glu or ATP solutions were used to record reversal potentials for ideally cation-selective membranes and the VDAC-doped membranes, electrode asymmetries and liquid junction potentials influence both sets of measurements in the same way. For the gramicidin experiments, 2 μ l of 0.01 μ g/ml gramicidin in ethanol was added to the *cis* side. Results are expressed as mean \pm SD.

MEASUREMENTS OF THE BULK DIFFUSION COEFFICIENTS

The diffusion coefficients of ATP, T-Glu, HPTS and BAPTA in bulk solutions were obtained using the method described previously (Rostovtseva et al., 2002). The method was based on measuring fluxes through a 0.45 μ m Millipore filter that separated two aqueous compartments containing 1.0 M NaCl solutions with 5 mM HEPES at pH 8.0. Thus, the salt solution used to measure the diffusion coefficients was the same as that used in the experiments with VDAC channels. The method was tested with molecules of known diffusion coefficient: glucose, sucrose, and raffinose. In order to minimize changes in the properties of the porous filter from experiment to experiment, the measurement of the flux of the compound of interest was performed simultaneously with the flux of sucrose, acting as an internal standard.

The sucrose content in the 50- μ l samples was determined as previously described (Dische, 1953) and by measuring the absorbance at 635 nm. The ATP concentration in the samples was checked by measuring the absorbance at 259 nm. The T-Glu con-

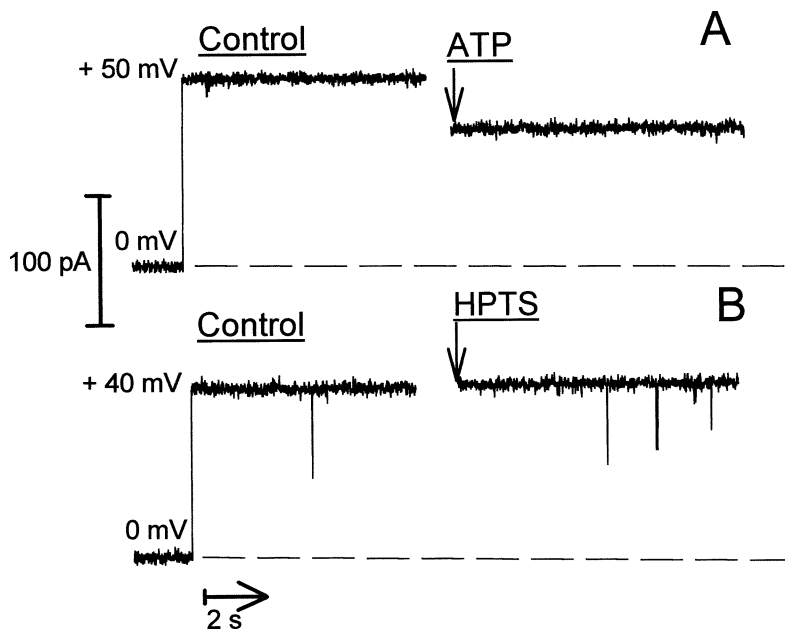


Fig. 2. Effects of ATP and HPTS addition on the current through single VDAC channels. 76 mM ATP decreased the mean current (*A*) but 70 mM HPTS caused no significant change (*B*). Each set of records was made on the same single channel and the same voltage was applied in control and treated records. The dashed lines show the zero-current level. The solutions contained 1.0 M (1.015 molal) NaCl and 5 mM HEPES pH 8.0 in the control, and this same solution was used to dissolve either ATP or HPTS (*see Methods*). Current records were not additionally filtered.

centration was measured using the ninhydrin method (Moore, 1968). The mixture of the sample with ninhydrin reagent (with 0.05% of glacial acid) in 1:3 v/v ratio was placed into sealed tubes under nitrogen. These mixtures were then heated in boiling water for 10 min. After cooling, 0.5 ml ethanol was added and the T-Glu concentration was determined from the absorbance at 570 nm. The concentrations of HPTS or BAPTA in the samples were checked spectrophotometrically. All assays were calibrated using the pure chemicals or published extinction coefficients.

The diffusion coefficients were calculated as previously described (Rostovtseva et al., 2002) by equating Fick's law to the rate equation:

$$Flux = DS \frac{\Delta c}{\Delta x} = k \Delta c v \quad (1)$$

where S is the area of the membrane, Δc is the concentration gradient, Δx is the effective thickness of the filter, and v is the volume on the *trans* side. The known diffusion coefficient for sucrose (Weast, 1979), $D_{sucr} = 0.521 \times 10^{-5}$ cm²/sec, was used to determine the ratio $S/\Delta x$, allowing D to be calculated from k .

ESTIMATION OF THE CHARGE OF THE NUCLEOTIDES AND T-GLU

At the high salt concentration used, some Na⁺ could bind to the negatively-charged solutes reducing their overall effective charge. The amount of free Na⁺ was measured with a sodium electrode (Model 86-11, Orion Research, Beverly, MA) in solutions of 100 mM of the test solute molecules. The solutions also contained the 1.015 molal NaCl at pH 8.0. The sodium electrode was calibrated with NaCl solutions of known molality and the presence of the test solute was assumed not to affect the activity coefficients. The total Na⁺ bound to the molecules was the difference between the free Na⁺ (Na⁺ electrode) and the total Na⁺ present. The latter included the Na⁺ from the NaOH used to neutralize the sample and any Na⁺ present in the reagent as purchased, assessed using a flame spectrophotometer (Model 560 Perkin-Elmer, Norwalk, CT) reading the absorbance at 589 nm. Subtracting the bound Na⁺ per mole from the known charge in dilute solutions yields the effective free charge on the solutes.

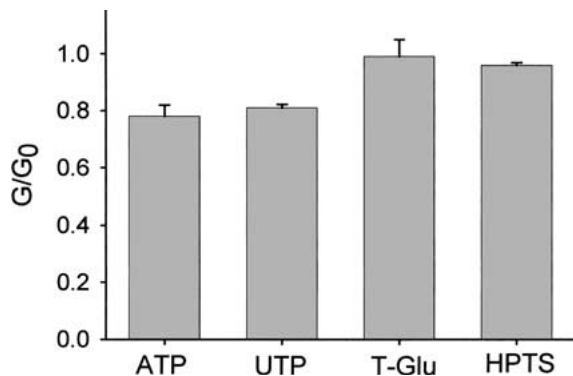


Fig. 3. The effects of 76 mM ATP, 98 mM UTP, 120 mM T-Glu and 70 mM HPTS addition on VDAC single-channel conductance. Conductances are expressed as ratios G/G_0 , to corresponding values in the solutions with no additions. The error bars are standard deviations.

Results

Permeation of molecules whose size is comparable to that of the pore of a membrane channel results in an interference with the flow of smaller ions (Krasilnikov et al., 1992; Bezrukov & Vodyanoy, 1993; Bezrukov, Vodyanoy & Parsegian, 1994; Bezrukov & Kasianowicz 1997; Bezrukov, 2000; Rostovtseva et al., 2002). This reduction in conductance can be used to detect the ability of these molecules to penetrate into and probably permeate through the channel. Previously we have shown that symmetrical addition of ATP resulted in a pronounced decrease of VDAC channel conductance in the presence of 1.015 molal NaCl (Rostovtseva & Bezrukov, 1998). Fig. 2*A* illustrates this effect. The left current trace shows a fully open channel at +50 mV applied potential and the right

Table 1. Bulk properties of the solutes used

	Amount of Bound Na ⁺	Molecular Weight With bound Na ⁺	Free Negative Charge	Diffusion Coefficient × 10 ⁻⁶ cm ² /sec
ATP ⁴⁻	2.6	563	-1.4	4.5 ± 0.3
UTP ⁴⁻	2.5	538	-1.5	-
T-Glu ⁴⁻	2.0	581	-2.0	4.1 ± 0.2
HPTS ³⁻	1.6	492	-1.4	4.8 ± 0.2
BAPTA ⁴⁻	2.7	534	-1.3	4.2 ± 0.1

trace shows the decrease of mean current through the same fully open channel after the NaCl bathing solutions were replaced by 1.015 molal NaCl solutions containing 76 mM ATP. Both solutions have virtually identical bulk conductivities and thus the reduction in single-channel conductance lies in ATP's interference with the current carried by small ions within the VDAC channel. This reduction could arise from a reduced mobility and/or accumulation of ATP in the channel. The concentration dependence of the conductance decrease is nonlinear, saturating at about 50 mM ATP. This saturation indicates a maximal occupancy of ATP within the channel.

Direct evidence that ATP permeates through the VDAC channel was obtained by measuring ATP fluxes through multichannel planar membranes using the luciferin/luciferase method (Rostovtseva & Colombini, 1996, 1997). Lacking the exquisite sensitivity of this method for other metabolites, the conductance reduction technique can be used as a general method to detect the ability of molecules of comparable size to ATP to penetrate into VDAC channels. Other mononucleotides, AMP, ADP, UTP, and GTP, and dinucleotides, NADH, NAD and NADPH, also penetrate into and likely permeate through VDAC channels because they all were shown to reduce the single-channel conductance to a very similar extent as ATP (Rostovtseva et al., 2002).

In contrast to nucleotides just mentioned, molecules of similar size and charge as ATP, but not found in the cytosol of cells, behaved quite differently. Fig. 2B demonstrates that addition of 70 mM HPTS to 1.015 molal NaCl does not change the mean current through fully open VDAC channels. This is in sharp contrast with the 20% drop in single-channel conductance measured in the presence of nucleotides such as ATP and UTP (Fig. 3). The same lack of the effect on channel conductance was observed for tetraglutamate (T-Glu, Fig. 3). No change in conductance indicates that the solution within the channel is still 1.015 molal NaCl and there is no HPTS or T-Glu in the channel to interfere with the flow of small ions.

An alternative explanation would require both T-Glu and HPTS to move through the channel as readily as they do in bulk. The conductivity of solu-

tions containing ATP, UTP, T-Glu or HPTS are all very close to the conductivity of the 1.0 M (1.015 molal) NaCl solution. Thus, if T-Glu and HPTS move through the channels as through bulk, one would expect no change in channel conductance from the added presence of these molecules. Thus, if VDAC channels were to interact more strongly with nucleotides than with HPTS and T-Glu, then perhaps this may explain the observed differences. However, such a large difference in permeation properties would need to originate in differences in structure.

The effective size and charge of T-Glu and HPTS were assessed by measuring their diffusion coefficient and net charge in the same salt solution used in the experiments with VDAC channels. The diffusion coefficients of T-Glu and HPTS (Table 1) show that the effective sizes of these molecules are very close to that of ATP. At the high salt concentration used, some Na⁺ could bind to the negatively-charged solutes reducing their overall effective charge. The amount of Na⁺ bound was determined by subtracting the free [Na⁺] (measured with a Na⁺ electrode) from the total [Na⁺]. Subtracting the moles of bound Na⁺ per mole of solute from their expected charge in dilute solutions yields the effective free charge on the solutes (Table 1). ATP, UTP and HPTS have a very similar free charge (-1.4 to -1.5), whereas T-Glu has a somewhat larger negative charge, -2.0. These differences seem too small to account for possible differences in permeation characteristics through VDAC channels.

An alternative method for assessing the ability of ions such as ATP and T-Glu to permeate through VDAC is to measure the reversal potential of a membrane containing VDAC in the presence of their gradient (Fig. 4). Experiments with a four-fold Na₄ATP gradient (200 vs. 50 mM pH 8.0) yielded -20.2 ± 0.4 mV. Gramicidin-containing membranes in the presence of the same Na₄ATP gradient had a reversal potential of -25.8 ± 0.9 mV. The -20mV reversal potential is in accord with Nernst-Planck calculations based on the measured 30-fold higher permeability of Na⁺ as compared to ATP.

Similar experiments were performed with a three-fold gradient of Na₄T-Glu (150 vs. 50 mM titrated to pH 8.0 with NaOH). The current record of one of

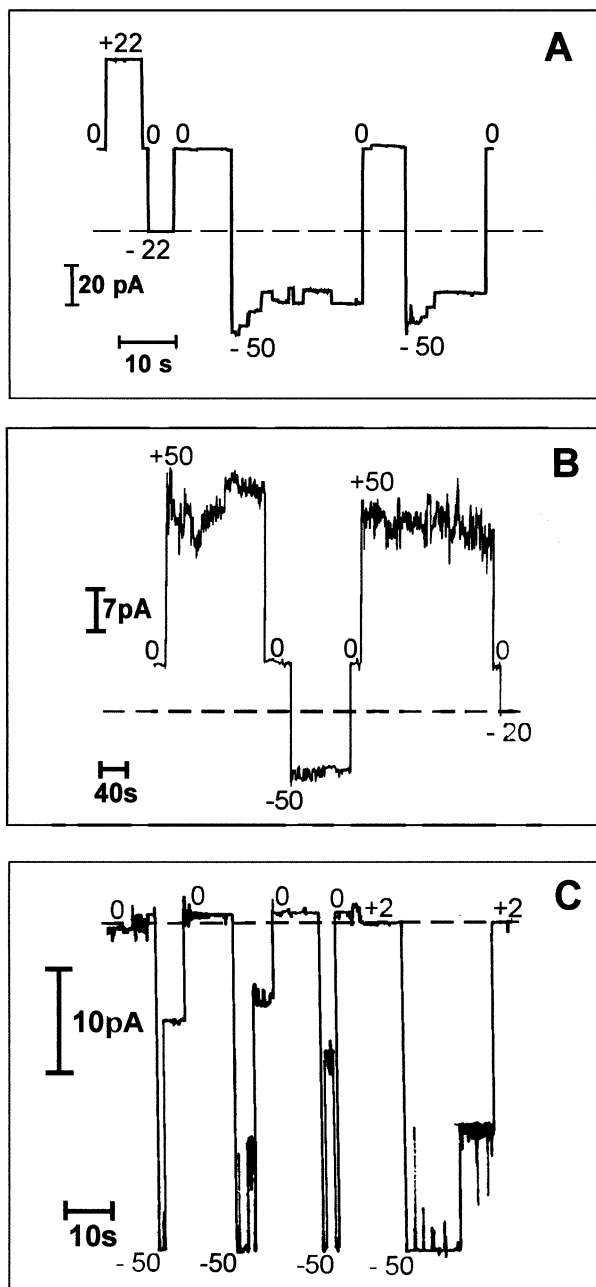


Fig. 4. Current records in the presence of a tetraglutamate or ATP salt gradients. (A) The gating of 3 channels at -50 mV applied potential in the presence of a four-fold gradient of ATP (200 mM *cis* and 50 mM *trans*) buffered with 5 mM HEPES and adjusted to pH 8.0 with NaOH. (B) The medium consisted of a threefold gradient of $\text{Na}_4\text{T-Glu}$ (150 mM *cis* and 50 mM *trans*). The reversal potential was -20 mV. (C) The medium consisted of a fourfold gradient of $\text{Na}_4\text{T-Glu}$ (plus equimolar TFA) sodium salt (187 mM *cis* and 47 mM *trans*) buffered with 5 mM HEPES at pH 7.4. The gating at -50 mV applied potential was pronounced and the reversal potential was close to 0 mV. The dashed lines indicate zero current. The numbers indicate the applied potential values.

these experiments is shown in Fig. 4B. The zero-current potential of VDAC was -19 ± 1 mV, which corresponded to almost ideal cation selectivity under

these conditions, as compared to -22.1 ± 0.6 mV for gramicidin. The difference is statistically significant but is small, indicating very little if any flux of T-Glu. Thus, the difference between the reversal potentials of VDAC and gramicidin measured in the presence of T-Glu is less than that with ATP. These results are inconsistent with the proposal that T-Glu flows through the channel faster than ATP and is too fast to interfere with the flux of small ions.

However, even a cursory look at Fig. 4B reveals that the VDAC channel current records are very noisy and that the channel does not close at ± 50 mV applied potential. This uncharacteristic behavior can be understood if one assumes that T-Glu drives the channel into its "closed" state.

Under the conditions used in these experiments, the inability of T-Glu to enter the channel should produce an enormous Donnan potential, positive inside the pore, that would drive the voltage sensor out of the channel (*see* Song et al., 1998b for the gating mechanism). Thus, the channels observed may indeed be in a closed state, which for VDAC is a lower conducting state with cation selectivity. Direct evidence for this interpretation was obtained in experiments with a small amount of permeant salt.

Experiments were performed in the presence of a four-fold gradient of $\text{Na}_4\text{T-Glu}$ (187 vs. 47 mM titrated to pH 7 with NaOH) but containing equimolar amounts of Na^+ trifluoroacetate $^-$ (TFA^-). The presence of low molecular-weight anions will greatly reduce the Donnan potential. The current record in Fig. 4C shows normal gating of a VDAC channel at 50 mV applied potential. Thus, the high concentration of T-Glu alone was probably not responsible for the uncharacteristic behavior of VDAC. With TFA^- present, the zero-current potentials of VDAC channels in the open state varied from -2 to $+1.5$ mV (compare with -29.3 ± 1.5 mV in the presence of gramicidin). Thus the currents carried by TFA^- and Na^+ compensated for each other and were essentially the same, confirming that TFA^- easily penetrates the channel.

VDAC gating is also evident in the presence of the ATP gradient (Fig. 4A). The closure steps are small because VDAC closure only blocks ATP flux and does not alter Na^+ permeability very much, thus only a small drop in current is expected. The somewhat higher reversal potential is often seen in multi-channel membranes and is attributed to the presence of a closed channel(s).

Attempts to measure the reversal potential of VDAC channels in the presence of an HPTS gradient failed because of detrimental effects of HPTS on VDAC at high HPTS concentrations in the absence of NaCl.

The surprising result that VDAC channels can select among molecules of similar size and charge is further substantiated by observations of open-chan-

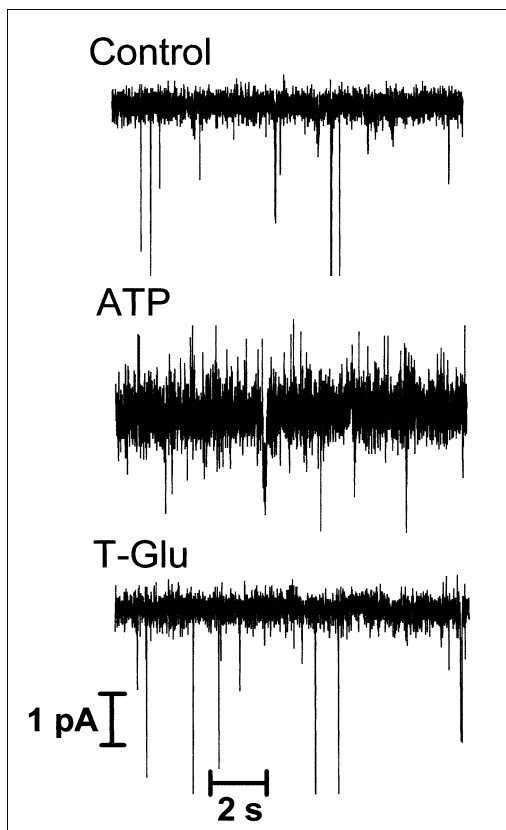


Fig. 5. Ion currents through the open single VDAC channels as altered by ATP and tetraglutamate addition. Conditions were as in Fig. 2. The recordings represent single channels without additions (*upper traces*), after addition of 76 mM ATP (*middle trace*), and after addition of 62 mM T-Glu (*lower trace*) at +50 mV applied potential. Current records were filtered using an averaging time of 3 msec.

nel current noise. Previous reports showed that mono- and dinucleotides containing the adenine base induced excess current noise through a fully open VDAC channel resulting from nucleotide binding within the channel (Rostovtseva & Bezrukov, 1998; Rostovtseva et al., 2002). Fig. 5 shows fragments of current traces through fully open VDAC channels in the presence of either 76 mM ATP or 62 mM T-Glu along with a control trace. T-Glu does not induce measurable noise above the control, but ATP produces significantly higher noise compared to control.

The spectral densities of the current noise showed that the amplitude of the noise is independent of frequency (i.e., white noise) between 100 and 1,000 Hz. The low-frequency spectral density, $S(0)$, was obtained by averaging the noise over this range. The results shown in Fig. 6 therefore represent increase in noise above the control level resulting from the presence of 76 mM ATP, 98 mM UTP, 120 mM T-Glu or 70 mM HPTS in the medium.

The ability of these molecules to induce noise is very different, in spite of their similar size and charge

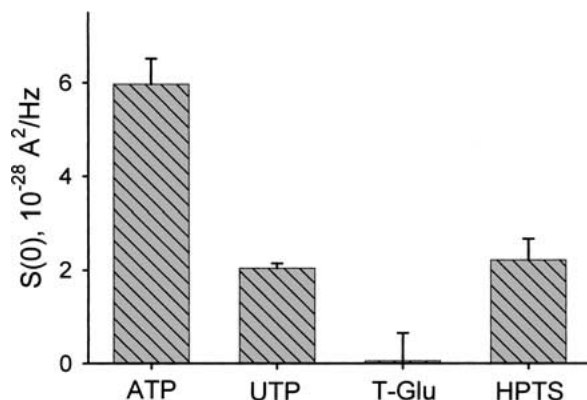


Fig. 6. The average excess current noise through a single VDAC channel in the presence of different solutes. The conditions were the same as in Fig. 3. The low-frequency current spectral density, $S(0)$, is the excess above control (no additions). The $S(0)$ value represents an average over the frequency range $100 < f < 1,000$ Hz at +50 mV with background subtracted (noise at 0 mV). The values for ATP and UTP were corrected for the reduced conductance induced by these nucleotides. The error bars are standard deviations.

(Fig. 6). The difference observed with ATP and UTP has been attributed to the selectivity of the noise-inducing binding site (Rostovtseva et al., 2002). The fact that T-Glu does not induce any measurable excess noise is consistent with the conclusion that it cannot penetrate into the channel. HPTS did increase noise to the same extent as UTP despite not reducing the single-channel conductance. These apparently conflicting results can be reconciled if HPTS were to increase current noise without penetrating into the channel.

If entry of HPTS into the channel were required to generate excess current noise then asymmetrical addition of HPTS should result in a voltage-dependent HPTS concentration profile in the channel and therefore a voltage-dependence of noise generation. In a previous work (Rostovtseva et al., 2002) asymmetrical addition of β -NADH to a VDAC-channel-containing membrane resulted in β -NADH-induced noise that depended on the sign and magnitude of the applied voltage. The average spectral density measured at different voltages was recalculated to 50 mV, because the low-frequency noise, assumed to be generated by conductance fluctuations unperturbed by the electrical field, is expected to increase as the square of the applied voltage (De Felice, 1981; Kasianowicz & Bezrukov, 1995; Rostovtseva & Bezrukov, 1998). After this correction, any changes in excess noise as a function of voltage should be attributable to changes in the local β -NADH concentration.

In Table 2 the voltage dependence of the noise level is expressed as the correlation coefficient of the relationship between measured noise and applied voltage. We defined the sign of the correlation coef-

Table 2. Correlation coefficients of the voltage dependence of current noise under asymmetrical conditions

<i>Cis/trans</i> conditions	Correlation Coefficient	
	Positive applied potential	Negative applied potential
55 mM NADH/0	-0.998	+0.991
70 mM HPTS/0	+0.688	+0.761
62 mM T-Glu/0	-0.546	-0.882
0/58 mM NADH	+0.997	-0.985
0/70 mM HPTS	-0.221	-0.395

ficient as positive, when the applied potential drives the negatively-charged permeant molecule into the channel and as negative, when the applied potential drives it out. When NADH was added to the *cis* side (55 mM NADH/0 in Table 2) the correlation coefficient approached +1 at negative applied potential and -1, at positive applied potential. In the cases of 70 mM HPTS or 62 mM T-Glu added to the *cis* side, the calculated correlation coefficients were outside the 95% significance level for both polarities of the applied potentials. Note that the sign of the correlation coefficients for experiments with both HPTS and T-Glu did not correlate with the sign of the applied potential. In the experiments when β -NADH was added to the *trans* side (Table 2) the sign of the correlation coefficients reversed with the sign of the applied potential. For β -NADH, the results are consistent with noise generation being dependent on concentration of nucleotide in the channel. For HPTS there was no such correlation (Table 2), indicating that noise generation does not depend on HPTS penetrating into the VDAC channel.

Discussion

Experiments with synthetic anionic molecules of similar size and charge as ATP yielded results consistent with the conclusion that these molecules are excluded from VDAC channels. The presence of T-Glu and HPTS did not reduce the conductance of VDAC channels, as would be expected if these were capable of penetrating into the lumen of these channels. The diffusion constants and the net charge of ATP, T-Glu and HPTS in the medium used for the experiments are very close (Table 1). Thus, the effective size and charge of these molecules cannot explain the difference in apparent ability to penetrate into VDAC channels. If correct, this result flies in the face of contemporary thinking regarding the factors that determine permeation through channel-forming large aqueous pores.

This surprising result was supported by VDAC selectivity measurements for Na^+ over T-Glu. The reversal potential values were almost the same as those observed with gramicidin, indicating a virtual

impermeability of T-Glu. The slightly lower reversal potential may indicate a finite T-Glu permeation through the channel and/or may have arisen from small amounts of anionic contamination that would not affect gramicidin. Recall that the reversal potential is a log function of the permeability ratio, and so it does not take much to move the measured value of the potential away from ideality.

Unfortunately, the reversal potential measurements are suspect because the properties of the channels under the measurement conditions were not characteristic of VDAC in the open state. VDAC closure results in movement of the positively-charged voltage sensor out of the channel lumen, resulting a net cation preference (Song et al., 1998a). Indeed, in the closed state that is easily accessible electrophysiologically, while the conductance of the channel to small ions drops to about half, the channel is impermeant to ATP (Rostovtseva & Colombini, 1996, 1997). Thus, the selectivity measurements in the presence of T-Glu may actually reflect the properties of the closed state. If so, one must ask, why are the channels closed? The presence of an impermeant polyvalent anion (T-Glu) would cause an extremely large Donnan potential, positive in the channel lumen. This potential would drive the sensor out of the lumen resulting in channel closure. Such a potential would be very sensitive to the ionic strength of any permeant salt. The presence of equimolar amounts of Na^+ TFA⁻ in the T-Glu solution restored normal gating behavior, as expected from Donnan potential theory.

In contrast to the lack of gating in T-Glu, gating was observed in the ATP reversal-potential experiments, indicating a qualitative difference between ATP and T-Glu, penetration of the former and exclusion of the latter.

The results from open-channel current-noise measurements provide further support for this conclusion for both T-Glu and HPTS. T-Glu did not induce the excess current noise, indicating no disturbance of small ion flow through VDAC. HPTS did produce a low level of noise. However, it is likely that the noise observed was due to a nonspecific effect, not to HPTS permeation into the channel. Since there was no significant voltage dependence of the HPTS-induced noise when HPTS was added asymmetrically, it is likely that the HPTS that induced the noise was outside the region of the applied transmembrane electric field, i.e., outside the lumen of the channel. Perhaps this noise arose from HPTS-VDAC protein interactions resulting in motions of the protein that caused time-dependent changes in ion flow through the channel. It should be mentioned that HPTS had a destabilizing effect on the channel. In each experiment, about 10 minutes after the introduction of HPTS, the properties of the VDAC channels degraded (large, irregular conductance fluctuations). The recordings were performed

before this happened. When additional VDAC channels spontaneously inserted into the membrane from the HPTS-containing bathing solution, the channels always displayed the degraded behavior. Thus, while undefined, the excess noise observed with HPTS is likely unrelated to HPTS penetration into the channel.

The big question raised by this work is, how can VDAC achieve selectivity that goes beyond the size and net charge of the permeating species. Based on non-electrolyte permeability and electron microscopic images, the radius of the pore is between 1.2 and 1.5 nm. Yet, the species being selected have a Stokes-Einstein radius of 0.4 to 0.5 nm. Clearly, the distribution of interactive sites inside the VDAC channel must be important. If one examines the evolutionary conservation of charged residues lining the pore of VDAC proteins from mammalian, fungal and plant sources, one finds remarkable conservation of specific groups at specific locations (Song & Colombini, 1996). Sometimes, even substitution with residues of the same charge (e.g., K for R) seems not to be tolerated. If net charge was all that was important, such conservation does not make sense. However, if the location of key charged and polar residues has been evolutionarily selected to achieve an electrostatic surface landscape optimized for passage of the highly-charged adenine nucleotides, such conservation makes sense. Additionally, other molecules with a surface charge distribution that is incompatible with that of the channel may face an exclusionary energy barrier. This is likely the case for T-Glu and HPTS.

Fig. 7 shows the structure of ATP obtained by MacroModel 4.5. The upper structures show front and side views of a superimposed set of likely conformations, while the lower structures are one of these conformations selected from the midrange. Note the asymmetric nature with the negatively charged phosphates on top of a ball-shaped region and the rather flat aromatic base extending in the bottom like the keel of a boat. Even without a 3-dimensional representation, Fig. 1 shows that HPTS must have a much more symmetrical charge distribution, and the same is likely true for the highly flexible tetraglutamate. The mismatch between the distribution of charges of these molecules and ATP may explain why they are unable to penetrate into the VDAC channel.

Another available molecule with size and charge similar to that of ATP is the fluorescent Ca^{2+} chelator, BAPTA. Experiments with BAPTA indicated that BAPTA could penetrate into the VDAC pore. Unfortunately, while we were able to obtain reasonable results after adding BAPTA to one side of a VDAC-containing membrane, two-sided addition resulted in severe degradation of the properties of

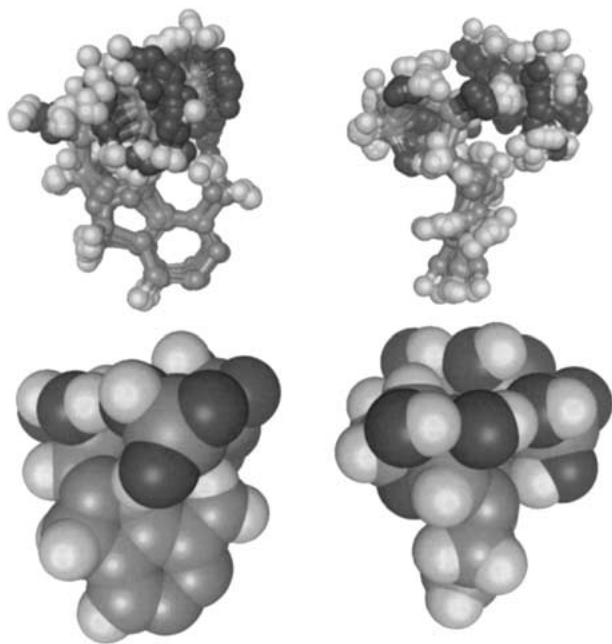


Fig. 7. The three-dimensional structure of ATP as calculated by MacroModel 4.5. The upper structures are front and side views of a set of 11 likely conformations of ATP. The bottom structures are front and side views of space-filling models of one of the conformations.

VDAC. One-sided addition showed a BAPTA-induced decrease in VDAC-channel conductance, indicating penetration into the channel. This very tentative observation is still consistent with the message of this paper, because BAPTA's structure, Fig. 1, is quite asymmetrical in a way that resembles ATP.

The ability of VDAC to display a level of selectivity generally attributed to narrow channels or carriers indicates that permeation of charged molecules through channels may be strongly influenced by the details of the protein landscape forming the wall of the pore. This is especially true for the charge distribution because electrostatic effects extend over long distances, especially inside cylinders (Zambrowicz & Colombini, 1993). Since adenine nucleotide flux through the mitochondrial outer membrane can be rate limiting, it is likely that selection pressure has optimized this protein landscape to favor rapid flux of these nucleotides. It is also likely that the structure of other large channels has been optimized to facilitate the transport of the substances they need to translocate. Maltoporin is a good example in this regard (Benz, 1988; Schirmer et al., 1995; Bezrukov, Kullman & Winterhalter, 2000). However, in general, whenever molecular surfaces must pass each other, even without making direct contact, electrostatic interactions likely generate some form of molecular friction that can be large or small depending on how the two surfaces match electrostatically. This notion clearly has implications beyond membrane channels.

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