ATP Transport Through a Single Mitochondrial Channel, VDAC, Studied by Current Fluctuation Analysis

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ABSTRACT The "molecular Coulter counter" concept has been used to study transport of ATP molecules through the nanometer-scale aqueous pore of the voltage-dependent mitochondrial ion channel, VDAC. We examine the ATP-induced current fluctuations and the change in average current through a single fully open channel reconstituted into a planar lipid bilayer. At high salt concentration (1 M NaCl), the addition of ATP reduces both solution conductivity and channel conductance, but the effect on the channel is several times stronger and shows saturation behavior even at 50 mM ATP concentration. These results and simple steric considerations indicate pronounced attraction of ATP molecules to VDAC's aqueous pore and permit us to evaluate the effect of a single ATP molecule on channel conductance. ATP addition also generates an excess noise in the ionic current through the channel. Analysis of this excess noise shows that its spectrum is flat in the accessible frequency interval up to several kilohertz. ATP exchange between the pore and the bulk is fast enough not to display any dispersion at these frequencies. By relating the low-frequency spectral density of the noise to the equilibrium diffusion of ATP molecules in the aqueous pore, we calculate a diffusion coefficient $D = (1.6–3.3)10^{-11}$ m^2/s. This is one order of magnitude smaller than the ATP diffusion coefficient in the bulk, but it agrees with recent results on ATP flux measurements in multichannel membranes using the luciferin/luciferase method.

INTRODUCTION

The transport properties of transmembrane channels are generally addressed in terms of mono or divalent ion conduction (Hille, 1992). The modern picture of ionic transport through excitable membranes is based on results obtained with new biochemical, pharmacological, and biophysical methods. Experiments with single ion channels using the patch-clamp technique (Neher and Sackmann, 1976) and channel reconstitution into planar bilayers (Bean et al., 1969) were especially helpful. Now, with progress in small signal detection and analysis, new approaches are being developed that allow us to study transport of polymers and high molecular weight metabolites at the single-channel level.

Analysis of fluctuations in ion current through a fully open single channel provides unique possibilities for obtaining additional information on ion and polymer transport. It was first successfully applied in studies of monovalent ion transport through the gramicidin A channel (Heinemann and Sigworth, 1989, 1990). Recently the method of current fluctuation analysis was used in studies of the transport of nonelectrolyte polymers (Bezrukov et al., 1994, 1996; Parsegian et al., 1995), and single-stranded RNA and DNA fragments (Kasianowicz et al., 1996) through single ion channels of different origin reconstituted into planar phospholipid membranes.

Received for publication 24 November 1997 and in final form 11 February 1998.

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0006-3495/98/05/2365/09 $2.00

Experiments of this kind may be useful in understanding the function of channels in protein transport. Indeed, strong evidence for the important role of transmembrane channels in the transport of biopolymers, such as proteins (Simon and Blobel, 1991) and transcription factors (Bustamante et al., 1995) is rapidly emerging. Attention is focused on targeting systems, insertion mediators, protein translocation motors, and protein folding mechanisms on the *trans* side of the membrane (Dietmeier et al., 1997; Schatz, 1997; Schatz and Dobberstein, 1996). The transport systems are believed to be universal and general; that is, able to import or export many different proteins. However, the physical properties of postulated protein-conducting channels are poorly understood (Schatz and Dobberstein, 1996; Martoglio et al., 1995; Görlich and Rapoport, 1993).

The voltage-dependent anion channel, VDAC (mitochondrial porin) is known to be responsible for most of the metabolite flux across the mitochondrial outer membrane. Experiments on intact mitochondria indicate that VDAC provides a pathway for nucleotide transport across the mitochondrial outer membrane (Benz et al., 1988; Liu and Colombini, 1992; Gellerich et al., 1993; Lee et al., 1994). By using the luciferin/luciferase method it was recently shown (Rostovtseva and Colombini, 1996, 1997) that VDAC is sufficient to mediate ATP flux through the mitochondrial membrane. In the fully open conformation its ATP permeability is 1.1×10^{-20} m^3/s, whereas in the closed state the permeability drops at least by two orders of magnitude; suggesting that VDAC is able not only to mediate, but also to control ATP efflux from mitochondria.

When reconstituted into planar phospholipid membranes, VDAC forms large aqueous pores (Colombini, 1994) that are open at low applied voltages (10–20 mV) and exhibit

weak anion selectivity in the open state. A working model of the VDAC pore (Colombini et al., 1987; Mannella et al., 1989) envisages a barrel with a diameter of 2.4–3.0 nm composed from a tilted α-helix and a β-sheet.

Based on these observations we consider VDAC in the presence of ATP to be an attractive model for studies of transport of high molecular weight polyions through protein transmembrane channels. Using the concept of a "molecular Coulter counter" (Bezrukov et al., 1994), we estimate partitioning of ATP between bulk solution and VDAC pore to give the free energy of ATP entrapment. By analyzing single-channel conductance and fluctuations in ion currents through an open channel in the presence of ATP we calculate the average number of ATP molecules and their diffusion coefficient within the VDAC pore.

These calculations indicate an attraction between ATP molecules and the pore. We also demonstrate that for reasonable hydrostatic pressure differences and electric fields $<10^7$ V/m, equilibrium diffusion dominates over hydrodynamic flow or electro-drift as the mechanism of ATP pore-bulk exchange. Otherwise, the effect of a single ATP molecule on channel conductance is surprisingly close to that expected for a macroscopic particle in a macroscopic capillary (DeBlois et al., 1977), but with particle and capillary sizes scaled down to angstroms. Thus we show that the mesoscopic VDAC pore is a Coulter counter with the added features of attraction and diffusion.

MATERIALS AND METHODS

VDAC channels were isolated from *Neurospora crassa* mitochondrial outer membranes and purified according to standard methods (Mannella, 1982; Freitag et al., 1983). Bilayer membranes were formed from monolayers made from a 1% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Inc., Alabaster, AL) in hexane (Aldrich Chemical Company, Inc., Milwaukee, WI) on a 70-μm diameter aperture in the 15-μm thick Teflon partition that separated two chambers (modified Montal and Mueller technique, 1972). The total capacitance was 60–70 pF and the film capacitance was close to 35 pF. Aqueous solutions of 1 M NaCl and 1 mM $CaCl_2$ were buffered by 5 mM HEPES at pH 8.0–8.3. All measurements were made at $T = (23.0 + 1.5)$°C.

Single-channel insertion was achieved by adding 0.1–0.2 μl 1% Triton solution of purified VDAC to 1.6 ml aqueous phase on one side of the membrane (*cis* compartment) while stirring. After a single channel was inserted and its parameters were recorded, membrane-bathing solutions in both compartments were replaced by ATP-containing solutions. In this way the ATP effects were observed on the same channel: the procedure that greatly reduced the scatter in datapoints related to variation in the open channel conductance. Dry ATP disodium salt (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 M NaCl aqueous calcium-free solution containing 5 mM HEPES. The ATP solution was adjusted to pH 8.0 with 1.5–2.0 M NaOH. This pH value was chosen for two reasons. First, at pH 8.0 virtually all ATP molecules exist in ATP^{4-} form [pK values for two terminal phosphate groups of ATP equal to 6.95 and 4.06 (Alberty, 1968). According to our titration curve, which have been measured for 100 mM disodium salt of ATP in 1 M NaCl, pK shifts to even lower values.] Second, at pH higher than 7.5 there is no interference with H^+-induced current noise of the VDAC open state (Rostovtseva et al., 1997).

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 determine ATP concentrations in the experimental chamber. Therefore, the ATP concentrations shown in the graphs correspond to the actual ATP concentrations in the experimental chamber after perfusion.

The membrane potential was maintained using Ag/AgCl electrodes in 3 M KCl, 1.5% agarose bridges assembled within standard 200 μl pipette tips (Bezrukov and Vodyanoy, 1993). Potential is defined as positive when it is greater at the side of protein addition (*cis*). The current was amplified by a Dagan 3900 integrating patch-clamp amplifier (Minneapolis, MN) in a mixed RC mode with a 3902 headstage and recorded by a PCM recorder (Vetter Co., Inc., Rebesburg, PA) operated in pulse code modulating mode. Recorded data were then analyzed using a personal computer and eight-pole Bessel or Butterworth filter (Frequency Devices, Haverhill, MA). Noise analysis of the open channel current was done according to the procedure described by Bezrukov and Vodyanoy (1993). Power spectral density was measured after the signal was filtered by a Butterworth filter, the corner frequency of which was set to ⅜ of the sampling frequency. An eight-pole low-pass Butterworth filter is well-suited for spectral measurements, as it provides a maximally flat amplitude response in the pass-band with a sharp roll-off at 48 dB per octave above the corner frequency. Fourier transformations were done on 2048 point vectors. The membrane chamber, headstage, and source of the applied voltage were isolated from external noise sources by a double high μ-metal screen (Amuneal Corp., Philadelphia, PA).

RESULTS

Fig. 1 illustrates the current through a single VDAC channel in the absence and presence of ATP in the membrane bathing solution. There are two effects induced by ATP addition: a decrease in the mean current and an increase in

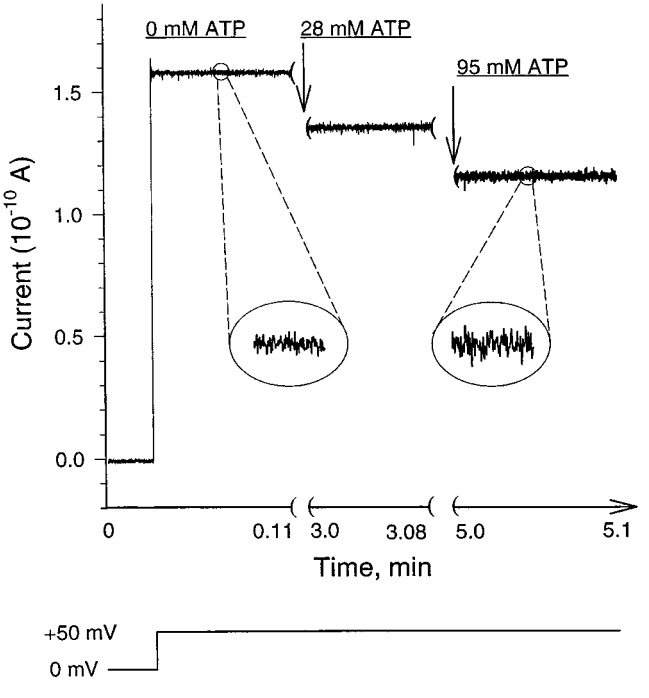

FIGURE 1 Ion currents through a single VDAC channel as altered by ATP addition to 1 M NaCl buffer solution. The recordings are taken from the same channel under the following conditions (*left* to *right*): without ATP at 0 mV and +50 mV applied potential; after addition of 28 mM ATP at +50 mV; after addition of 95 mM ATP at +50 mV. The bottom trace indicates the potential applied across the membrane. The insets show the current recording at a finer scale. Note that with increasing ATP concentration the mean current decreases while the current noise grows. Current recordings are filtered by a low-pass 8-pole Bessel filter at 1.2 kHz.

the current noise. The records correspond to the fully open channel. It is known (Colombini, 1989) that conductance of the open VDAC channel varies within 2–5% of the mean value. For example, in our experiments the channel conductance in 1 M NaCl is 3.4 ± 0.1 nS. Therefore, to measure the ATP-induced conductance changes with an appropriate accuracy, the effect of ATP in each experiment was evaluated comparing conductance of the same channel in 1 M NaCl ATP-free and in ATP-containing solutions. In the experiment illustrated in Fig. 1 the initial bathing solution was replaced by ATP-containing solutions in the order of increasing ATP concentration. The parts of the recordings corresponding to the solution perfusions (~3 min) were cut out for the clarity of illustration.

Fig. 2 shows the relative changes in the conductance of a single VDAC channel and in the conductivity of bulk solution as functions of ATP concentration. The changes of bulk electrolyte conductivity reflect both a negative effect of ATP interfering with Na^+ and Cl^- ion conductivity and a positive contribution from the conductivity of ATP^{4-} anion itself (see Discussion). It is seen that the negative effect of ATP interference dominates in the high sodium chloride concentration used in our measurements.

The ATP-induced decrease in channel conductance is much more significant than in bulk solution conductivity. It is also nonlinear starting from 50 mM ATP concentrations on. This pronounced reduction may point to an attractive interaction between VDAC pore and ATP molecules resulting in ATP accumulation within the pore.

To obtain kinetic information on ATP transport through the channel, we have analyzed noise of the open channel current. It is important to note that all measurements were performed for the VDAC open state; the noise of the closed states has completely different features and is not considered in the present paper. Fig. 3 illustrates the current spectral density for a single VDAC channel in the presence of 95 mM ATP at +50 mV applied potential (*top trace*) versus the background at 0 mV (*bottom trace*). The relatively high conductance of a single VDAC channel (3.4 nS) contributed significantly to the level of the background noise. In the absence of the channel the noise level of an unmodified membrane (*dashed arrow* in Fig. 3) is several times lower than the noise in the presence of the channel at 0 mV.

The current noise spectral density averaged over the frequency range between 100 and 1000 Hz is shown in Fig. 4 for different ATP concentrations. To account for the equilibrium (Johnson) contribution to the noise signal, the noise spectrum from the open channel at 0 mV was subtracted from the noise spectrum obtained at 50 mV applied potential. It is seen that addition of ATP increases the open channel noise with a maximal effect in the vicinity of 80 mM ATP. Without any ATP added there is a measurable current noise of the open channel, $S(0) = (1.61 \pm 0.19) \times 10^{-28}$ A^2/Hz. Interestingly, even small amounts of ATP (5 mM) induce measurable current noise, while the effect on conductance is far too small to be detected at these low ATP concentrations.

Fig. 5 shows the dependence of the low-frequency spectral density on applied voltage at two ATP concentrations. The ATP-induced noise is plotted against voltage squared to visualize possible deviations from quadratic law expected for conductance fluctuations whose dynamics do not depend on applied voltage (DeFelice, 1981). It is seen that up to 80

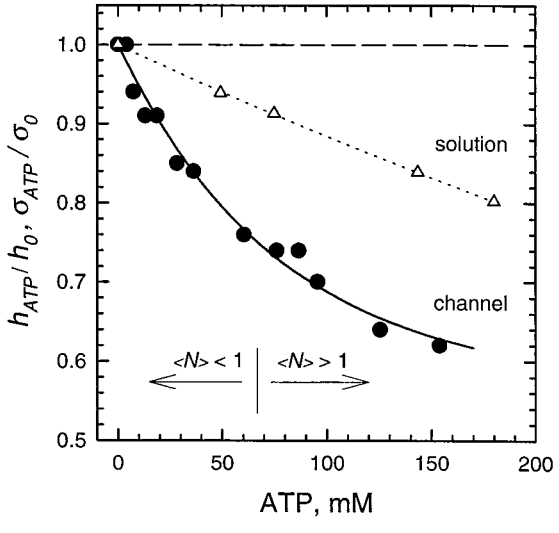

FIGURE 2 The effect of ATP addition on VDAC channel conductance and bulk solution conductivity. Conductance, h_{ATP}, and conductivity, σ_{ATP}, in the presence of ATP are given as ratios to corresponding values in ATP-free solutions, h_0 and σ_0. The solid line illustrates the fitting to Eq. 1. The horizontal dashed line corresponds to the unchanged channel conductance. The dotted line represents a linear regression through experimental points. The vertical dash indicates the ATP concentration corresponding, on average, to one ATP molecule per volume of solution equal to that of the VDAC pore.

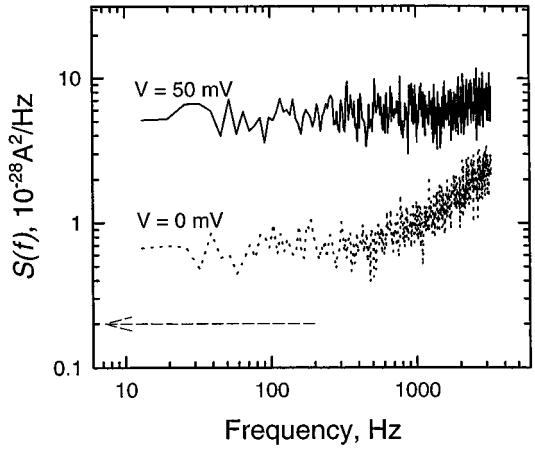

FIGURE 3 The power spectral density of current noise of a single open VDAC channel in the presence of 95 mM ATP at +50 mV transmembrane potential (*upper trace*) in comparison with the background noise obtained at 0 mV (*lower trace*). It is seen that the spectrum of the ATP-induced noise is "white" at these frequencies. The increase in the spectral density of the background at high frequencies comes from the input amplifier and electrolyte noise. The horizontal arrow corresponds to the noise level in the absence of a channel.

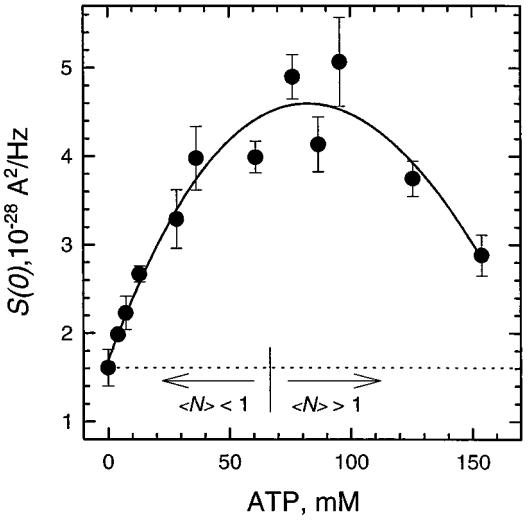

FIGURE 4 The low-frequency spectral density, $S(0)$, as a function of ATP concentration. Each point represents spectral density, as illustrated in Fig. 3, averaged over the range $100 < f < 1000$ Hz with the background subtracted. The solid line is drawn to guide an eye through experimental points. Spectral density level without ATP is represented by the horizontal dashed line. The applied potential was +50 mV.

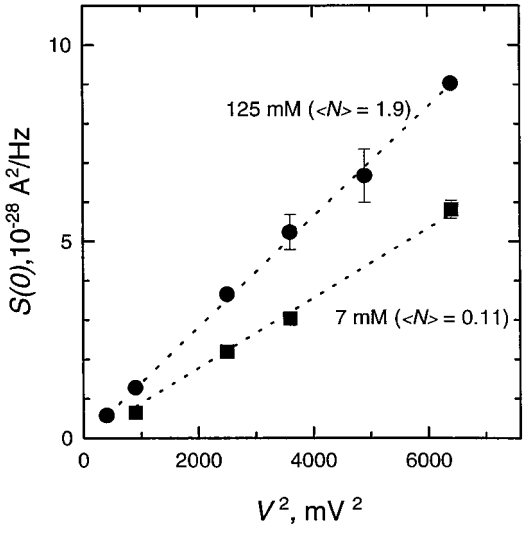

FIGURE 5 The low-frequency noise spectral density, $S(0)$, as a function of the applied voltage squared. It is seen that the low-frequency noise induced by 7 mM and 125 mM of ATP is proportional to the voltage squared. Such a proportionality is expected in the case when the system's conductance fluctuation dynamics are not perturbed by the applied electric field (DeFelice, 1981). $\langle N \rangle$ indicates the average number of ATP molecules per volume of solution equal to that of the VDAC pore.

mV of transmembrane potential the noise is well-described by quadratic dependencies for both small and high ATP concentrations.

MODEL

To analyze the obtained results, we will further explore the concept of an ion channel as the "molecular Coulter counter" (Bezrukov et al., 1994) that was inspired by work of Charles Bean and his colleagues with submicron diameter Nuclepore capillaries (DeBlois and Bean, 1970; DeBlois et al., 1977). The resistive pulse principle that has been used in Coulter counters since 1953 is based on the fact that nonconducting particles suspended in electrolyte solution decrease its conductivity. When such a particle moves from a bulk solution into a small capillary, it reduces the capillary conductance. If the particle suspension is dilute enough and the particles are sufficiently large, this temporary reduction in the capillary conductance can be easily detected and particle size and velocity extracted (Allen, 1967). In the model below we apply this approach considering the VDAC aqueous pore as a capillary and the ATP molecule as a particle.

The several orders of magnitude size difference between a capillary in a standard Coulter counter and an aqueous pore of an ion channel (microns versus angstroms) leads to certain difficulties in applications of the well-developed particle sizing method to ion channels. The geometrical considerations successfully used for calculating conductance, and thus sizing, in Coulter counters (DeBlois et al., 1977) can be utilized only with great caution for particles of several angstroms. Fortunately, the particle "blocking effect" on conductance can be determined experimentally by measuring specific conductivity of electrolyte solutions with well-defined particle concentrations. Still, problems related to particle-pore interactions and possible peculiarities of ion transport through ion channel pores both in the absence and presence of such particles could complicate the interpretation.

In the absence of any interactions and disregarding all steric effects, the average number of ATP molecules, $\langle N \rangle$, that occupy VDAC channels at a given ATP bulk concentration is $\langle N \rangle = n\nu$, where n is the number density of ATP molecules in the bulk solution and ν is the channel pore volume. Using the following pore dimensions—length $L = 5$ nm, diameter $d = 2.5$ nm—we obtain 67 mM for the bulk ATP concentration that would correspond to one ATP molecule in the channel (*vertical dash* in Fig. 2).

What is the change in VDAC ionic conductance, Δh_s, expected from a single ATP molecule entering the channel? First, from Fig. 2 one can see that the increasing content of ATP decreases both channel conductance and solution specific conductivity. The ATP molecule slows small-ion currents more effectively than it contributes (as a polyion) to the conductivity of 1 M NaCl solutions. This effect depends on NaCl concentration and in sufficiently diluted salt solutions is reversed (see Discussion).

The channel conductance curve in Fig. 2 shows greater sensitivity to ATP than the bulk conductivity does. Such behavior can indicate attraction between ATP and the VDAC pore and/or show different efficiency in ion current blocking by ATP in the bulk compared to that in the channel. A single exponential analysis of the channel conductance (*solid line* in Fig. 2) indicates a saturation to 0.57

of the initial ATP-free value

$$\langle \Delta h \rangle / h_0 = 0.43\{1 - \exp(-0.013[\mathrm{ATP}])\}, \qquad (1)$$

where $\langle \Delta h \rangle$ is the ATP-induced channel conductance reduction and [ATP] is ATP concentration in mM.

Fig. 6 gives a schematic illustration of the VDAC channel in the lipid bilayer with the channel and ATP dimensions shown approximately to scale. From steric considerations, one can see that the maximum occupation of the channel is limited to ~2–4 molecules, $N_{\max} = 2–4$. An occupancy of 4 corresponds to dense packing of ATP inside the pore, taking into account physical size of the molecule plus ~3Å of hydration water; an occupancy of 2 corresponds to the case when ATP molecules are predominantly oriented in the pore by the ATP/pore interaction. Thus the 0.43 saturation in $\langle \Delta h \rangle / h_0$ is reached when VDAC pore has ~2–4 ATP molecules. This observation allows us to estimate the relative conductance change per one ATP molecule as 0.11–0.22. This range for the effect of a single ATP molecule on pore conductance compares well with the value expected from the ATP-induced reduction of bulk solution conductivity (see Discussion).

To calculate the ATP diffusion coefficient, we use a formula describing open channel noise generated by a nonconducting particle's equilibrium exchange between the channel pore and bathing solution (Bezrukov et al., 1994). This formula relates the particle diffusion coefficient inside the pore, D, with a low-frequency "white" part of the power spectral density of the current noise, $S_i(0)$

$$D = (\Delta h_s)^2 L^2 \langle N \rangle V^2 / 3 S_i(0), \qquad (2)$$

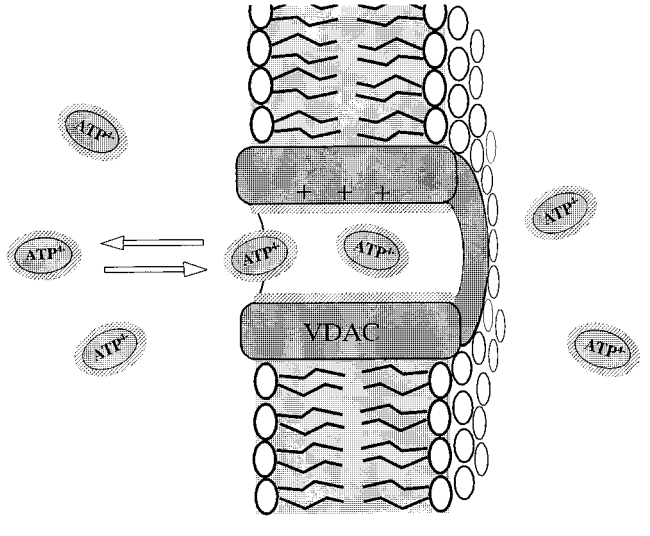

FIGURE 6 Schematic illustration of the VDAC pore in the presence of ATP. The open channel is a barrel composed predominantly of a cylindrical β-sheet wall. Cartoon shows the relative sizes of the open VDAC channel and ATP molecules together with the layers of hydration water shown as shadows. ATP molecules in the bulk solution are in a dynamic equilibrium with those inside the channel.

where L is the pore length and V is the transmembrane voltage. Parameters Δh_s and $\langle N \rangle$ describing the change in the pore conductance due to the entrance of one particle and the average number of particles in the pore, correspondingly, may be combined to reduce the number of variables that cannot be measured directly. Indeed, for small $\langle N \rangle$ we have $\langle \Delta h \rangle = \Delta h_s \langle N \rangle$, that is, the total ATP effect on the channel conductance equals the conductance reduction induced by a single ATP molecule times their average number in the channel pore. Hence, we have

$$D = \Delta h_s \langle \Delta h \rangle L^2 V^2 / 3 S_i(0), \qquad (3)$$

where $\langle \Delta h \rangle$ is measured directly. The result of the diffusion coefficient calculation still depends on Δh_s, but Eq. 3 shows now that this dependence is only linear (not quadratic, as it superficially appears in Eq. 2).

For the reasons stated above, we apply Eq. 3 in the region of small ATP concentrations where all observed effects are linear in ATP concentration, thus suggesting small channel occupancy $\langle N \rangle$. We use the following parameters: $L = 5 \times 10^{-9}$ m, $V = 5 \times 10^{-2}$ V, $\Delta h_s = (0.11–0.22) \times 3.4\ 10^{-9}$ S, $\langle \Delta h \rangle = 2.1 \times 10^{-10}$ S (12 mM ATP, Fig. 2), $S_i(0) = 1.0 \times 10^{-28}$ A^2/Hz (12 mM ATP, Fig. 4). Introducing these values in Eq. 3 we arrive at: $D = (1.6 - 3.3) \times 10^{-11}$ m^2/s. This value is an order of magnitude smaller than diffusion coefficient of ATP usually reported for bulk water solutions (c.f. 3×10^{-10} m^2/s, Deihl et al., 1991) but compares well with the data obtained on multichannel VDAC membranes. Measured using the luciferin/luciferase method, the diffusion coefficient of ATP through VDAC channels was found to be equal to $(2.8–7.2) \times 10^{-11}$ m^2/s (Rostovtseva and Colombini, 1997). The two-fold difference may account for the difference in experimental conditions: the luciferin/luciferase ATP flux measurements were performed with 0.1 M salt solutions, while the present study was carried out with 1.0 M NaCl. The higher salt concentration stabilized the channel in its open conformation by reducing its voltage sensitivity (data not shown). As explained in the next section, high salt concentration also increased the "conductivity contrast" of ATP molecules.

To conclude, we note that an order of magnitude drop in diffusion coefficient of ATP in the pore as compared to the bulk is exactly what could be expected from "restricted diffusion" considerations (Bean, 1972) for the macroscopic particles and pores with these relative sizes. In this respect, ATP transport through the VDAC channel significantly differs from sugar transport through the LamB channel. In that case, strong binding that decreased sugar translocation rate by many orders of magnitude was detected by noise analysis performed on multichannel bilayers (Nekolla et al., 1994).

DISCUSSION

We have demonstrated that the analysis of current noise from a single open channel can be used to characterize the

transmembrane transport of high molecular weight polyions. The "molecular version" of the Coulter counter used in the present work differs in size from a standard version by several orders of magnitude. Such a significant difference in size must induce certain qualitative changes in the system properties. Here we discuss three size-related issues: 1) nonconducting particle versus polyion concepts; 2) diffusion versus electro-drift and flow as mechanisms for relaxation of concentration fluctuations in the pore, and 3) particle partitioning and ATP-pore electrostatic interaction.

Nonconducting particle versus polyion

When a macroscopic nonconducting spherical particle of radius r enters a pore of radius R and length L filled with a conducting fluid of conductivity σ_0, and condition $r \ll R$ holds, the conductance of the pore is decreased by an amount

$$\Delta H_s = 2\pi\sigma_0 r^3/L^2, \tag{4}$$

that does not depend on pore radius. This result follows directly from the expression for the resistance pulse height, $2r^3/\pi\sigma_0 R^4$, used for particle sizing in standard Coulter counters (Gregg and Steidley, 1965; DeBlois and Bean, 1970; DeBlois et al., 1977).

Interestingly, Eq. 4 permits us to calculate particle size using solution conductivity data of the type shown in Fig. 2. Consider a macroscopic (e.g., centimeter range) cylindrical pore of radius R and length L with initial conductance $H = \pi R^2\sigma_0/L$ that will accommodate $\langle N\rangle = \pi R^2 L n$ particles upon their addition to the conducting fluid to the number density n. When n is small, nonconducting particles will produce an additive effect so that a total decrease in the pore conductance will be equal to $\langle\Delta H\rangle = \Delta H_s\langle N\rangle$. It is clear that the relative effect, $\langle\Delta H\rangle/H$, does not depend on the pore shape, and thus can be measured by a standard conductometer as a reduction in solution conductivity, $\Delta\sigma/\sigma_0$. Therefore, equating these two values, we obtain the following expression for the particle radius

$$r = \sqrt[3]{\frac{1}{2\pi n}\frac{\Delta\sigma}{\sigma_0}}. \tag{5}$$

It is tempting to apply this result for the particles of molecular size. ATP molecules have dual features concerning their influence on solution conductivity. First, at neutral pH they bear four charges and induce additional conductivity. Second, they are large enough to interfere with currents produced by small ions (e.g., Na^+ and Cl^-). These two features act in opposite directions and, in close analogy with small-angle neutron scattering methods, may be "contrasted" by changing small-ion solution conductivity.

To illustrate this idea, we have measured the change in conductivity of sodium chloride solutions induced by ATP addition. Fig. 7 presents the relative effect, $\Delta\sigma/\sigma_0$, as a function of salt concentration. All samples were prepared the same way as 1 M NaCl solutions used in experiments with VDAC: dry ATP disodium salt was added together with 1.5–2.0 M NaOH (for acidity adjustment to pH 8.0) to NaCl solutions to obtain final ATP concentration of 50 mM. At ~0.5 M NaCl the addition of ATP does not change solution conductivity. The electric "visibility" of ATP addition can be "contrasted" by diluting or concentrating NaCl solutions. Fig. 7 shows that at smaller salt concentrations, ionic features dominate; ATP and sodium counterions increase conductivity. At higher salt concentrations, ATP molecules act as nonconducting particles that decrease solution conductivity.

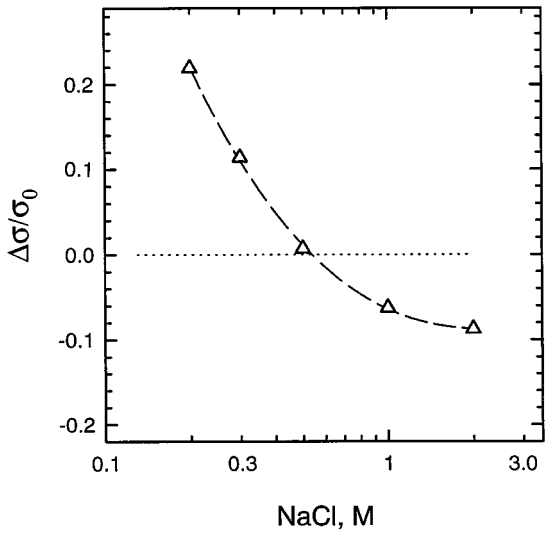

FIGURE 7 The magnitude and sign of the effect of 50 mM ATP addition on solution conductivity, σ, depends on salt concentration. In concentrated NaCl solutions, ATP interferes with small ion currents introducing a blocking effect that reduces solution conductivity. However, for small and moderated salt concentrations, each ATP molecule increases conductivity by adding one polyion bearing four negative charges plus four Na^+ counterions. The dashed line is a third-order regression through experimental points.

Formal application of Eq. 5 to the 2 M NaCl point (that, in our sample preparation procedure, corresponds to virtually unchanged small ion concentration before and after ATP addition) gives $r = 0.77$ nm, in accord with the hydrodynamic radius deduced from the diffusion coefficient in bulk water (Diehl et al., 1991). Thus, an ATP molecule in concentrated salt solutions seems to act as a nonconducting particle of this size. This observation agrees well with the conclusions derived from a study of sodium dodecyl sulfate (SDS) micellar solutions (Bezrukov et al., 1992). Analysis of conductance fluctuations generated by micellar flow through a macroscopic capillary showed that at high NaCl concentrations 10-nm SDS spherocylinders could be treated as nonconducting particles with (negative) conductance contribution calculated from Eq. 4 with corrections for micellar shape and polydispersity.

At 1 M NaCl concentration used in our experiments with VDAC, the nonconducting particle effect is already partly screened by ATP ionic properties and increased small ion concentration: $\Delta\sigma/\sigma_0$ is reduced in comparison to 2 M

NaCl. Using Eq. 4 for $r = 0.77$ nm and the pore of 5 nm length filled with 1 M NaCl aqueous solution, we can calculate the expected conductance change introduced by a single ATP molecule as $\Delta h_s = 0.89$ nS. This value corresponds to $\Delta h_s/h_0 = 0.26$ and somewhat exceeds $\Delta h_s/h_0$ estimates (0.11–0.22) that we deduced from the channel conductance curve in Fig. 2. Such deviation is to be expected since, due to electroneutrality, an ATP molecule entering the channel not only blocks current paths but also increases number of mobile Na^+ ions in the pore that decrease ATP blocking effect.

In summary, the change in channel conductance produced by an entry of one ATP molecule, estimated from channel conductance, is in reasonable agreement with the value calculated for macroscopic particles with scaled dimensions. This agreement may indicate that the parameters of "small ion" transport through the mesoscopic aqueous VDAC pore do not differ much from those in macroscopic objects. The electrostatic effects in channel-mediated ion transport (Green and Andersen, 1991; Chen and Eisenberg, 1993; Green and Lu, 1995; Eisenberg, 1996; Lu and Green, 1997) induced by ATP charges are probably minimized by screening in concentrated salt solutions used in the single-channel measurements of the present study.

Flow, drift, flux, and diffusion

The only dynamics that were taken into account in our noise spectra derivation are those induced by an equilibrium diffusion-driven exchange of particles between the ion channel pore and external solution. In standard Coulter counters suspended particles are always driven by the combined effects of applied hydrostatic pressure difference, electroosmosis, and electrophoresis (DeBlois et al., 1977). What are the contributions from these sources in our case?

The effect of hydrodynamic flow has been addressed previously (Bezrukov et al., 1994). Simple estimates show that for a pore of 5 nm length and 1 nm radius the Brownian motion of 0.5-nm particles dominates particle dynamics for hydrostatic pressure differences up to $\sim 10^8$ Pa. In other words, the diffusion-driven exchange of particles between the pore and membrane-bathing solution remains faster than the exchange by the flow until the pressure difference across the membrane is greater than a 10-km-high water column. This estimate does not mean, of course, that to measure flow-related effects one has to apply this pressure difference (e.g., see Rosenberg and Finkelstein (1978) who used osmotic pressures of 1.5–2.5 osmol/kg, equivalent to (3.5–6.0) $\times 10^6$ Pa, to measure electrokinetic effects in gramicidin channels); it only shows that the fluctuation relaxation and, therefore, the fluctuation spectrum will be governed by equilibrium diffusion for pressures $<10^8$ Pa.

In considering the effects of an applied electric field, we first note that the luciferin/luciferase method of ATP flux measurements has shown that the ATP flux through the VDAC channel is driven by applied electric fields starting from voltages of 10 or 15 mV (Table 1 of Rostovtseva and Colombini, 1997). This finding seems to contradict the noise versus voltage dependence reported in the present study. Fig. 5 displays a linear relation between $S(0)$ and V^2. This linearity suggests that the conductance fluctuations, and thus the dynamic behavior of ATP in the channel, are virtually undistorted by transmembrane voltages up to 80 mV. To resolve this apparent contradiction we estimate the influence of drift-induced dynamics on fluctuations by comparing the time of diffusional relaxation with the time it takes an ATP molecule to pass the pore drifting down electric potential.

Using the Einstein relation for particle mobility, $u = D/kT$, where k is the Boltzmann constant and T is the absolute temperature, we estimate the time for a particle to pass a pore of length L by drifting in an electric field,

$$\tau_{\text{drift}} \approx kTL^2/4eDV, \tag{6}$$

where $4e$ stands for four elementary charges of the ATP molecule at neutral pH. Taking $V = 30$ mV and $D = 2 \times 10^{-11}$ m^2/s, we have $\tau_{\text{drift}} \approx 3 \times 10^{-7}$ s. To compare this time with the characteristic time of particle diffusion through the pore from one side of the membrane to the other, we use

$$\tau_{\text{trans-diffusion}} \approx L^2/D \tag{7}$$

to obtain $\tau_{\text{trans-diffusion}} \approx 1 \times 10^{-6}$ s. Thus, drift in electric field is faster at 30 mV transmembrane voltage and this mechanism is dominating ATP transport.

The characteristic time that accounts for current fluctuation temporal behavior and defines the current noise bandwidth is, however, much shorter and can be estimated by (Feher and Weissman, 1973):

$$\tau_{\text{diffus.relaxation}} \approx L^2/12D \tag{8}$$

or even by $L^2/6\pi D$ (Bezrukov et al., 1994) using a slightly different definition of relaxation time. Both estimates give $\tau_{\text{diffus.relaxation}}$ of $\sim 1 \times 10^{-7}$ s. Therefore, with drift in electric field already dominating transmembrane transport at 30 mV of applied voltage, the characteristic times of the processes form the following succession: $\tau_{\text{trans-diffusion}} > \tau_{\text{drift}} > \tau_{\text{diffus.relaxation}}$.

This time hierarchy reconciles the apparent contradiction with the results of ATP flux measurements. Specifically, at $V = 30$ mV, relaxation of fluctuations in particle number inside the channel due to diffusion is faster than particles' exchange due to drift in electric field, even though the net directional flow through the channel at this voltage is already dominated by the drift (Rostovtseva and Colombini, 1997).

ATP partitioning and interaction with the channel

The transition from micron-to-nanometer-sized pores and particles may also bring about significant electrostatic or electrodynamic interactions that are negligible in standard

Coulter counters. Four elementary charges carried by the ATP molecule at neutral pH make such interactions feasible. Our data suggest the existence of attractive interactions between ATP and the VDAC pore even at the high salt concentration, 1 M NaCl, used in our channel experiments, and thus strongly support the evidence for ATP binding to VDAC that has already been reported by others (Flörke et al., 1994).

First, the ATP-induced effect on channel conductance saturates with increasing ATP concentration (Fig. 2) and indicates repulsive interactions between ATP molecules inside the channel. This repulsion significantly influences partitioning of ATP into the pore. At bulk ATP concentrations of 100 mM, repulsion reduces the effect expected from the linear extrapolation at small ATP concentrations by about twofold. The average distance between molecules in the bulk solution for 100 mM, $l \approx n^{-1/3} \approx 2.5$ nm, is too large for such a pronounced effect in 1 M NaCl. Therefore, based on this qualitative behavior, we argue that the channel effectively "concentrates" ATP within its pore, promoting such interactions.

Quantitatively, using simple geometrical arguments for hard sphere partitioning into a cylindrical pore (e.g., Colton et al., 1975), the partition coefficient, $p_g(r, R)$, is

$$p_g(r, R) = (1 - r/R)^2. \quad (9)$$

The ratio of the Stokes-Einstein radius of ATP [$r = 0.7$ nm, calculated from the ATP diffusion coefficient in water of 3×10^{-10} m^2/s (Deihl et al., 1991)] to the pore radius R is ~0.6. According to Eq. 9 this gives 0.16 for the equilibrium partitioning coefficient and, thus, for the expected mean occupancy of the pore at 67 mM of ATP.

Comparing this prediction for ATP partitioning in the absence of interaction to the partitioning obtained from our data, we can estimate the characteristic energy of ATP-pore attraction, F_{attr}. For the partition coefficient accounting for this attraction we write

$$p = p_g(r, R)\exp(F_{attr}/kT). \quad (10)$$

According to our analysis, at 67 mM ATP the VDAC pore is occupied by about one-half of the number of ATPs at saturation, i.e., by one or two ATP molecules. This gives $p \approx 1.0$–2.0 for the concentration region where the ATP effect is still rather linear (Fig. 2), so that the repulsion between ATP molecules can be ignored. Substituting this result into Eq. 10 with $p_g = 0.16$, we have $F_{attr} \approx 1.8$–2.5 kT per ATP molecule.

CONCLUSIONS

Analyzing ATP-induced effects on the mean current and current noise of the fully open VDAC channel we demonstrate:

- In concentrated salt solutions ATP molecules entrapped in a several-nanometer diameter aqueous pore of the VDAC channel show electrical and dynamical properties that are surprisingly close to the properties expected for nonconducting macroparticles scaled to the corresponding nanometer size.
- The diffusion coefficient of ATP in the VDAC pore is an order of magnitude less than its bulk diffusion coefficient. This finding agrees well with the restricted diffusion predictions for macroscopic particles and capillaries of similar relative sizes.
- The VDAC pore apparently concentrates ATP molecules within its confines. Attraction of ATP to the VDAC pore can be described by a characteristic energy of ~2 kT.

We are grateful to V. Adrian Parsegian for fruitful discussions and encouragement at all stages of this work. We thank Igor Vodyahoy, Marco Colombini, Donald C. Rau, and Helmut Strey for valuable suggestions and reading the manuscript. VDAC samples were generously provided by M. Colombini.

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