Published in final edited form as:
Biochim Biophys Acta. 2007 October ; 1768(10): 2510–2515.

VDAC closure increases calcium ion flux

Wenzhi Tan and **Marco Colombini**
Department of Biology, University of Maryland, College Park, MD, 20742.

Summary

VDAC is the major permeability pathway in the mitochondrial outer membrane and can control the flow of metabolites and ions. Therefore Ca^{2+} flux across the outer membrane occurs mainly through VDAC. Since both Ca^{2+} fluxes and VDAC are involved in apoptosis, we examined whether Ca^{2+} is required for channel formation by VDAC isolated from rat liver. The voltage gating of VDAC does not require Ca^{2+} and it functions normally with or without Ca^{2+}. Additionally, VDAC generally shows a higher permeability to Ca^{2+} in the closed states (states with lower permeability to metabolites) than that in the open state. Thus VDAC closure, which induces cell death, also favors Ca^{2+} flux into mitochondria, which can also lead to permeability transition and cell death. These results are consistent with the view that VDAC closure is a pro-apoptotic signal.

Keywords

PTP; mitochondria; apoptosis; voltage gating; swelling; planar membrane

Introduction

Mitochondria are the governors of both cell life (e.g. energy generation) and cell death. Some regulation of both of these functions occurs at the level of the outer membrane in that it controls the flow of metabolites and the release of intermembrane space proteins into the cytosol. These two functions are connected in that a drastic reduction in metabolite flow through the outer membrane, associated with VDAC closure*, can lead to protein release and apoptosis [1–2]. However, Ca^{2+} induced mitochondrial swelling and subsequent cell death may require an increase in Ca^{2+} flux and thus an increase in outer membrane permeability. Perhaps these apparently conflicting changes in outer membrane permeability may be understood if VDAC closure is irrelevant to a small ion such as Ca^{2+} and the Ca^{2+}-induced mitochondrial swelling is strictly an inner-membrane phenomenon.

It is generally believed that mitochondrial swelling is caused by the opening of PTP (permeability transition pores). The pore has a molecular mass cut-off of 1500 Da. Many have proposed that PTP involves both the inner and outer membrane through the participation of VDAC. However, knockout of VDAC1 does not seem to affect the formation of PTP [3],

Address reprints address to: Marco Colombini, Department of Biology, University of Maryland, College Park, MD 20742, Fax: 301 314-9358, Tel: 301 405-6925, Email: colombini@umd.edu.

*VDAC gating has evolved not to regulate the flow of K+ and Cl− ions, but to regulate the flow of metabolites. We use small ions because it is convenient to do so to follow the different conformational states of VDAC. However, one must think about VDAC from its physiological function, to allow metabolites, especially ATP and ADP to cross the outer membrane. Thus VDAC closure refers to closure to the flow of metabolites. The normal closed states of VDAC allow a substantial flow of small ions. The conductance to KCl is generally 40–50% of the open state conductance.

making the participation of VDAC in PTP questionable. Nevertheless, it is generally believed that Ca^{2+} should flow easily through VDAC channels because VDAC shows only a weak selectivity for small mono-valent ions [4–5]. Indeed, in a recent study [6], the authors concluded that VDAC opening promotes calcium flux into mitochondria followed by PTP and mitochondrial swelling, i.e., VDAC opening induces cell death.

This conclusion is consistent with the report that anti-apoptotic Bcl-2 family proteins close VDAC channels [7]. It is also in agreement with studies on gelosin, hexokinase, RuR, DIDS etc. [8–11] that closure of VDAC is an antiapoptotic signal but in sharp contrast to other findings that VDAC closure is a pro-apoptotic signal. For example, the anti-apoptotic protein, Bcl-x_L, favors the opening of VDAC channels [12–13], while the pro-apoptotic oligonucleotide, G3139 closes VDAC [2,14–15]. In addition, removal of required growth factors from cell lines leads to decreased MOM permeability to metabolites, such as phosphocreatine, and subsequent apoptosis. This is also consistent with VDAC closure. It is possible that apoptosis induced by VDAC closure is through a different pathway from calcium induced PTP.

In addition to the role of Ca+2 in apoptosis, Ca^{2+} is an important intracellular second messenger. At sub-micromolar concentration, it can also stimulate mitochondrial respiration and phosphorylation by the activation of dehydrogenases [16–17]. The flux of $Ca2^{+}$ into mitochondria seems come from the SR/ER-mitochondria contacts [18–19], which propagate calcium signaling into the mitochondria. The permeability of the mitochondrial outer membrane would thus have implications in other Ca^{2+} signaling besides mitochondrial swelling and cell apoptosis.

To clarify the role of VDAC gating in Ca^{2+} flux through the outer membrane, we examined the permeability of VDAC to Ca^{2+} in the open and closed states. The states with higher Ca^{2+} permeability are different from the state traditionally considered to be the open state.

Materials and Methods

Planar phospholipid membrane studies

Planar phospholipid membranes were generated according to standard methods [20–21]. The membrane was formed from phospholipid monolayers consisting of diphytanoyl phosphatidylcholine, polar extract of soybean phospholipids (both from Avanti Polar Lipids, Alabaster, AL) and cholesterol (Sigma, St Louis, MO) in a 1:1:0.1 mass ratio.

VDAC was purified from mitochondria isolated from rat liver [22–23]. In the ion selectivity measurements, a 0.1 μL aliquot of the VDAC-containing solution (2.5% Triton X100, 50 mM KCl, 10 mM Tris, 1 mM EDTA, 15% DMSO, pH 7.0) was stirred into 4–6 mL of aqueous solution containing 80 mM $CaCl_2$ aqueous (unless stated otherwise) solution on the *cis* side of the chamber. The *trans* side, containing 20 mM $CaCl_2$ aqueous solution (unless stated otherwise), was held at virtual ground by the voltage clamp. The solutions were unbuffered and no other ions were deliberately added. After a single channel insertion, a triangular voltage wave at a frequency of 3 mHz from −52 mV to +52 mV was applied to the *cis* side. The current was recorded at a rate of 1 ms per point with 2 kHz filtering. All experiments were performed at approximately 23°C.

Results

The voltage gating of VDAC channels is not affected by Ca^{2+}

Detergent solubilized VDAC was added to a solution containing no calcium ions (0.4mM EGTA was added to chelate any possible calcium contaminations) bathing a planar

phospholipid membrane (Fig. 1). VDAC channels inserted into the membrane and these events were recorded as increases in current. In the absence of calcium ions, VDAC can remain in the open state and closes in response to a 50 mV applied potential. The kinetics of the closure are indistinguishable whether in the absence or presence of 1.6 mM Ca^{2+} (Fig. 1). Moreover, the presence or absence of Ca^{2+} does not change the conductance of VDAC. In 1.0 M NaCl the conductance is 3.3 ± 0.3 nS (mean ± S.D.) in the presence of 0.1 mM EGTA and 3.4 ± 0.1 nS (mean ± S.D.) in the presence of 1 mM $CaCl_2$.

Measurement of the permeability of VDAC to Ca^{2+} and Cl^-

The permeability of VDAC to small ions includes Ca^{2+}, which was reported to permeate both the open [4] and closed states [24] (Rostovtseva, T.K., unpublished data) of VDAC. VDAC is the most abundant channel in the MOM (mitochondrial outer membrane). Thus its permeability to Ca^{2+} is very important as this may affect calcium homeostasis and cell death signaling. However, the value of the ion flux of Ca^{2+} through VDAC has never been directly and accurately determined. Here, $CaCl_2$ was used to measure the ion flow through VDAC.

The current carried by cations and anions are given by the GHK current equation (constant field equation):

$$I_s = P_s z_s^2 \frac{VF^2}{RT} \frac{a_{si} - a_{so}\exp(-z_s FV/RT)}{1-\exp(-z_s FV/RT)} \quad (1)$$

Where I_S is current carried by ion S, z_S is the charge of s, P_S is the permeability of VDAC to S, β_S is the activity of S (i denotes *cis* side, o denotes *trans* side), V is the potential difference across the membrane, F is the Faraday constant, R is the gas constant and T is the temperature. The single-ion activities were obtained from the salt activity coefficients. For $CaCl_2$, the activity coefficient is 0.5355 for 80 mM solution (*cis* side) and 0.6644 for 20 mM solution (*trans* side) [25].

At any given voltage, all the parameters except I_S and P_S are known in equation 1. Thus we can define:

$$X_s = z_s^2 \frac{VF^2}{RT} \frac{a_{si} - a_{so}\exp(-z_s FV/RT)}{1-\exp(-z_s FV/RT)} \quad (2)$$

which is a known parameter, thus

$$\sum Ps\,Xs = I \quad (3)$$

where I is the recorded current. If each X_S is different, equation 2 and equation 3 can be combined to fit the current voltage curve to generate the permeabilities to each ion. A four-fold gradient of $CaCl_2$ (with ionic strength in the physiological range) was used as mentioned in the Materials and Methods.

A single VDAC channel was used for the current-voltage measurements. The parameters were calculated by fitting to the current voltage curve. The slope at zero voltage is defined as the conductance of the VDAC channel, the zero-current intercept is V_{rev} (reversal potential) and the zero-pontential intercept is I_0 (zero potential current).

Fig. 2 shows an example of the fitting of equation 2 and 3 to a segment of a current-voltage record. The recorded current was divided into two currents: calcium current and chloride current. The permeabilities were directly obtained from the fitting. V_{rev} (reversal potential) and I_0 (zero potential current) for each state were determined by extrapolating the current-voltage curve to intercept the appropriate axis.

This method is more accurate than the calculation of ion permeabilities from a linear extrapolation of the current-voltage curve to the axes. The linear extrapolation is not justified because the current shows rectification.

In the open state, the conductance, G = 0.520 ± 0.005 nS, V_{rev} = 25.5 mV ± 0.2 mV, I_0 = −12.4 ± 0.1 pA (mean ± S.E., n = 15). In the positively closed states, G = 0.20 ± 0.02 nS, V_{rev} = 15 ± 2 mV, I_0 = ±2.8 ± 0.5 pA (mean ± S.E., n = 19). In the negatively closed states, G = 0.29 ± 0.05 nS, Vrev = 2 ± 3 mV, I_0 = 0 ± 1 pA (mean ± S.E., n = 15).

The distributions of conductance, V_{rev}, I_0 are very narrow for the open state and very broad for the closed states. This is consistent with previous observations [26–27], indicating the presence of multiple closed states. More interestingly, there is a clear difference ($P < 0.001$) between the states observed at negative and positive voltages. The anion preference drops in both cases as compared to that of the open state but states achieved at negative potentials are more favorable to Ca^{2+} flux.

Fig. 3 shows the fitted values of the permeability of VDAC to Ca^{2+} and Cl^- in the open state (the units are always 10^{-16} L·s^{-1}), P_{Ca} = 0.94 ± 0.05, P_{Cl} = 22.6 ± 0.2 (mean ± S.E., n = 15); in the positively closed states, P_{Ca} = 1.4 ± 0.2, P_{Cl} = 6.3 ± 0.9 (mean ± S.E., n = 19); And in the negatively closed states, P_{Ca} = 4 ± 1, P_{Cl} = 5 ± 1 (mean ± S.E., n = 15). Clearly, the VDAC channel is highly selective for chloride ions over calcium ions in the open state. When it is closed by high potentials, the permeability to chloride ions and calcium ions becomes comparable, especially in those states observed in the negative potential region.

There is a significant increase in Ca^{2+} permeability in both sets of closed states but in the negatively closed states the permeability is more that 4 times that of the open state (Fig. 4). In some states, the increase can be more than 10 times (Fig. 3).

Discussions

The gating of VDAC channels is not influenced by the presence of Ca^{2+} in the medium, but the flow of Ca^{2+} through VDAC is affected by VDAC gating. In its open state, VDAC is poorly permeable to Ca^{2+}. Extrapolating our results down to a more physiological Ca^{2+} concentration of 1 μM, the flux would be 20 ions per sec. This increases 4 fold when VDAC closes but in some states to as much as 10 fold (Fig 3,Fig 4). It is possible that some signals may lock VDAC in the more Ca^{2+} conducting states and expedite the Ca^{2+} flux.

In order to obtain accurate estimates for permeability and flux, one needs to have accurate values for activity coefficients. Unfortunately, only salt activity coefficients are available and thus we used these to convert concentrations into activities. However, one can estimate single-ion activity ratios by measuring the reversal potential for salt gradients using ion exchange membranes. Using the salt activity ratios, the calculated permeability ratio of chloride over calcium for the open state of VDAC is more than 25 fold, which is very close to ideal anion selectivity. The permeability ratio doubled if one uses the single ion activity ratios. Thus the calcium flux through VDAC in the open state is even lower, and therefore VDAC closure could enhance calcium flux even more than the value we report.

High-conducting states can be permeable to Ca^{2+}

We observed VDAC functional states that have a similar conductance as that of the open state, but with higher permeability to Ca^{2+} (Fig. 3). This is consistent with previous publications (e.g. [26]) and the “cationic state” of VDAC previously reported [28]. In our experiments, those states only occur rarely at high negative voltages. They are considered closed states because

they are expected to have reduced permeability to metabolites, most of which are anionic. In the Ca^{2+} experiments, these states occurred exclusively at negative potentials.

Ca^{2+} and VDAC gating

We observed no significant Ca^{2+} influence on the voltage gating property of VDAC. A possible reason for the difference between the results presented here and those of Báthori *et al.* [29] is the different methods used to make planar membranes. We use the monolayer method of Montal and Mueller [20] to generate solvent-free membranes. The hole in the partition that supports the planar membrane is coated with petrolatum (a hydrocarbon mixture ranging from C_{20} to C_{90}), then phospholipids in hexane are layered on the top of the buffer solution. Once the hexane has evaporated, the planar membranes are made by apposing monolayers from the two sides of the membrane. In contrast, Báthori *et al.* [29] made the planar membrane by directly painting a decane solution of phospholipids across the hole on the partition separating two sides of the chamber.

The major difference here is the solvent and its propensity to partition into the planar membrane. At room temperature, hexane, which has a vapor pressure of 0.172 atm [30], evaporates very quickly from the monolayers and thus does not contaminate the membrane. On the other hand, decane, with the vapor pressure of 0.00136 atm, two orders of magnitudes lower than that of hexane [30], evaporates much more slowly than hexane. In addition, the painting method for making planar membranes directly applies the decane solution to the hole, which has already been submerged into the buffer solution. Considering the virtual insolubility of decane in water, all the decane will remain with the lipids. Indeed, the decane annulus is necessary to allow the bilayer to interface with the much thicker plastic partition. Unlike the petrolatum coating used in the monolayer method, decane molecules are 5 times smaller and diffuse easily into phospholipid membranes. This solvent increases the membrane thickness by 1 nm, and may change such properties as the surface tension and fluidity of the membrane and affect the stability, conductance and voltage gating of VDAC. In addition, Ca^{2+} can interact with charged lipids and spontaneously thin the membrane, favoring channel formation, which may account for the regulation of Ca^{2+} on observed channel conductance. For gramicidin channels, an increase in membrane thickness (even due to the presence of decane) reduces the channel lifetime by orders of magnitude [31–33].

Báthori *et al.* [29] also reported that Ca^{2+} increases the rate of proton flow across the mitochondria outer membrane in permeabilized cells, consistent with their reported Ca^{2+} effect on VDAC channel formation. However, Ca^{2+} may act indirectly in permeabilized cells. In fact numerous publications report flux studies through VDAC reconstituted in liposomes in the presence of as much as 1 mM EDTA. In these studies, sugars and short polymers were shown to flow through VDAC [e.g. 34–35]. Also, the presence of Ca2+ does not increase the flux [fig. 5C of Ref. 8].

Ionic strength and VDAC permeability to Ca^{2+}

The permeability to Ca^{2+} measured here is very different from that reported by Gincel *et al.* [6]. They measured a P_{Ca}/P_{Cl} of 0.38 and claim a high flux of calcium through the open VDAC channel. This conclusion supports their hypothesis that the open state of VDAC favors Ca^{2+} flux, mitochondrial swelling and cell death. The main observed difference between their experiments and results shown here is the measured reversal potential in $CaCl_2$ gradients. Their observed reversal potential of 10 mV is less than half of what is measured here. This is most likely due to the difference in salt concentration (Table 1).

Assuming a linear concentration gradient inside the channel, the difference in $CaCl_2$ concentration between the two sets of experiments is 50 mM vs. 200 mM. The ionic strength

is 150mM vs. 600mM. Thus our ionic strength is at a physiological level. The difference in ionic strength translates into a difference in screening of charges within the channel. The calculated Debye Length at room temperature is:

$$\frac{1}{\kappa} = 0.3044 I^{-0.5} nm \quad (4)$$

Thus the Debye length is 0.78 nm for our experiments vs. 0.39 nm. Hence, in the higher ionic strength there are fewer electrostatic interactions between the positive charges in the inner wall of VDAC and Ca^{2+} ions permeating through the VDAC.

In addition to the difference in ionic solutions, Gincel *et al.* [6] did not compare the Ca^{2+} flow through VDAC in open and closed states. Perhaps, even under their conditions, the closed states of VDAC could be more permeable to Ca^{2+}.

The physiological impact of VDAC gating

In our observations, the permeability of Ca^{2+} through VDAC is different in the states observed at positive versus negative voltages (Fig 3,Fig 4). This clearly shows an oriented insertion of mammalian VDAC into planar membranes and an asymmetrical VDAC function. The impact of VDAC gating on the regulation of Ca^{2+} flux will depend on the orientation of VDAC in mitochondria and the sign of the potential across the outer membrane.

At present, there is no way of relating the orientation of VDAC in planar membranes and that in the mitochondrial outer membrane. However, it is still interesting to consider the impact of VDAC closure and selectivity change on the calcium flux. Fig. 5A shows the open probability of VDAC in response to voltages. By combining this information with the permeability of VDAC to Ca^{2+} in the different states, one can calculate the time-averaged permeability of VDAC to calcium in response to voltages. If we assume that VDAC in mitochondria is in the orientation, whose closure greatly favors calcium flux from the cytosol into mitochondria, the calcium flux in response to 1 μM cytosolic Ca^{2+} can be calculated (Fig. 5B).

Porcelli and coworkers [36] measured the pH difference between the mitochondrial intermembrane space and the cytosol. Considering the highly-buffered nature of these compartments, it is reasonable to conclude that protons will be at equilibrium between these two compartments and thus calculate the potential difference across the outer membrane. This turns out to be 40 mV negative potential in the intermembrane space compared to the cytosol [36]. Thus, from Fig. 5 there is a 3 fold increase of calcium flux if VDAC closes with a 40 mV potential. Considering that there are the VDAC modulator [37–38] and other proteins which may favor VDAC closure and may favor particular closed states, the actual effect could be even larger. Thus the closure of VDAC actually favors Ca^{2+} flux, even though the closed states has a smaller pore diameter (1.8 nm in the closed states compared to 2.5 nm in the open state) [39–40].

Accumulating evidence links VDAC gating with apoptosis [2,13]. It is clear that the open state of VDAC facilitates metabolite flow and thus maintains mitochondria in a healthy state, preventing cytochrome c release. In addition, in the open state, Ca^{2+} permeability is low. In the closed state, VDAC reduces metabolite flux, increases Ca^{2+} permeation, and thus sensitizes mitochondria to apoptotic signals. The gating process of VDAC is not only an interesting biophysical phenomenon, it also changes the properties of VDAC to initiate or be in harmony with changes in the physiological state of the cell.

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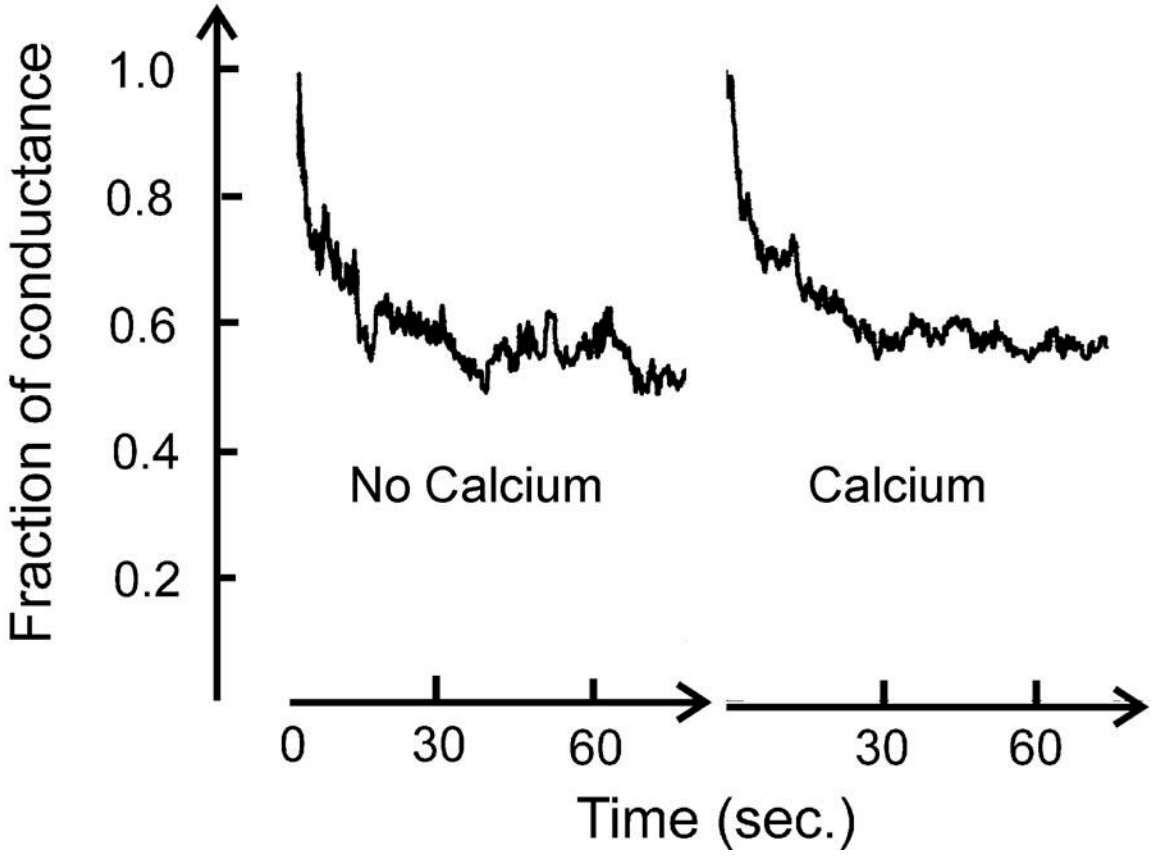

Fig. 1.

VDAC was reconstituted into a planar phospholipid membrane composed of 0.5% PC, 0.5% asolectin and 0.05% cholesterol in the presence of 1.0 M KCl, 20mM HEPES, pH=7.2. 0.4mM EGTA was then added to each side of the chamber to chelate any possible trace amount of Ca^{2+}. 2mM Ca^{2+} was added later to compare VDAC's gating in the presence and absence of Ca^{2+}. (Note: there is enough buffer to control the pH even after acid is released following chelation of some Ca^{2+}) VDAC's gating was recorded by applying 50 mV (cis side is ground). The total conductance in the absence and presence of Ca^{2+} is 115 nS and 179 nS, respectively. There is an 18-minute gap between these records and the conductance increased because of VDAC insertion. The percent conductance drop and the rate of decay are essentially the same with and without free Ca^{2+}.

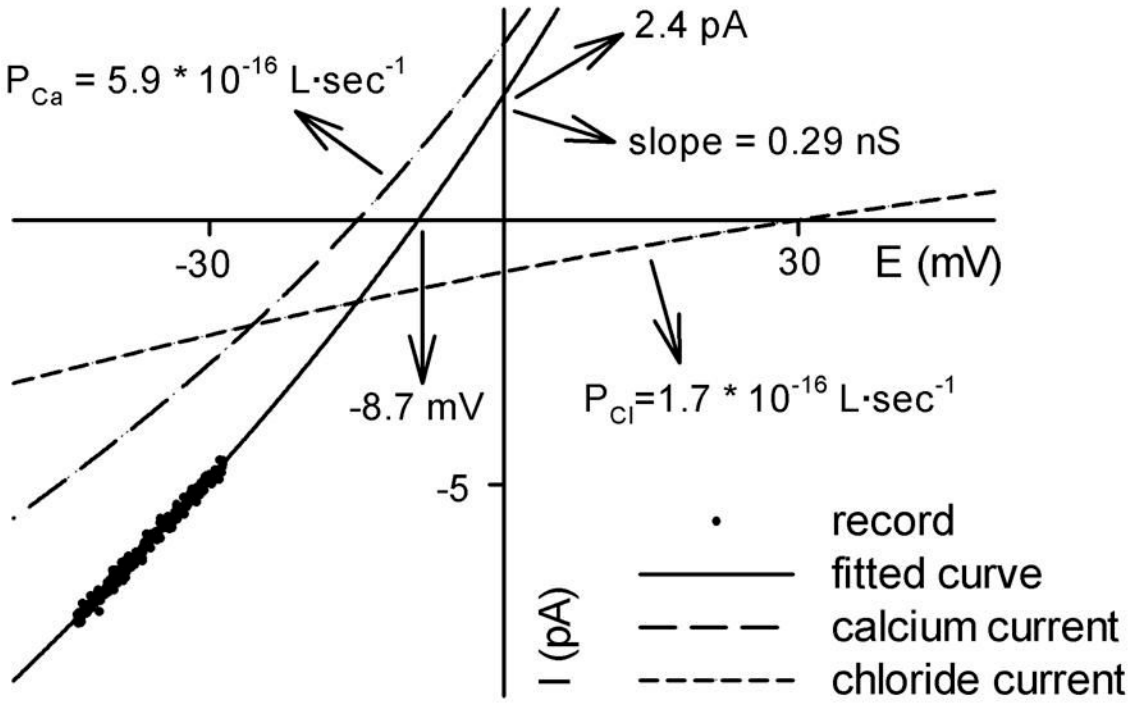

Fig. 2.

An example of fitting equation 2 and equation 3 to a segment of a single-channel current-voltage record representing one state of a VDAC channel (a closed state here). A four-fold gradient of $CaCl_2$ was present (see Materials and Methods). The arrows and associated numbers indicate the parameters obtained from the fitting.

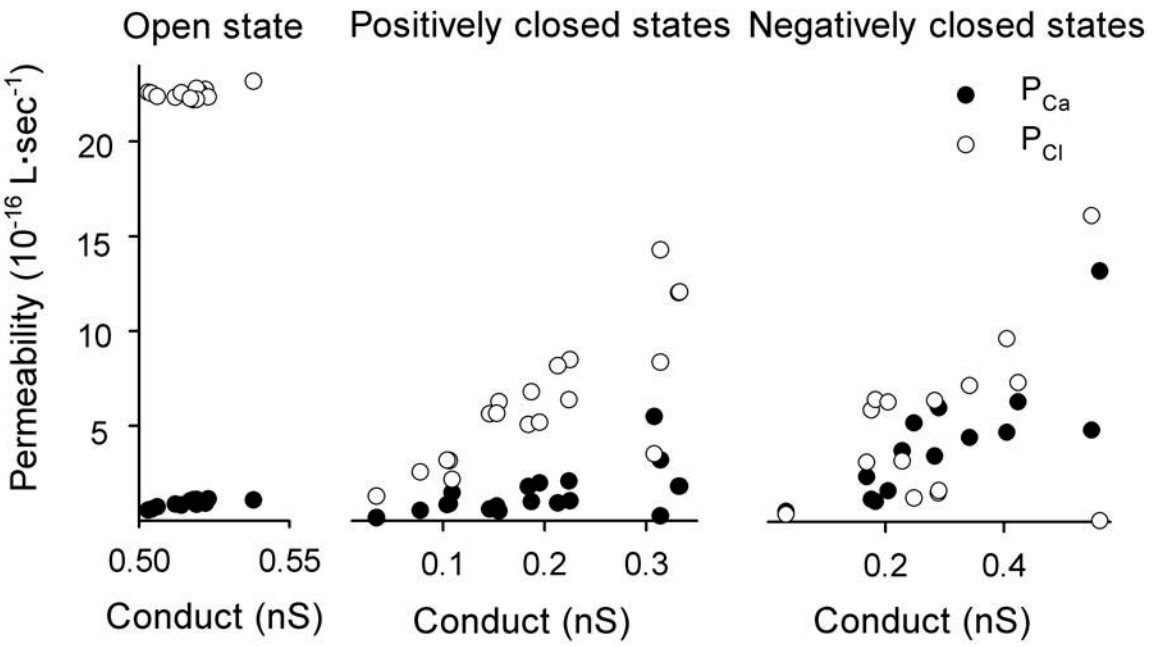

Fig. 3.
Permeability of VDAC to calcium and chloride ions as a function of the total conductance of the channel. Each value is obtained directly as in Fig. 2.

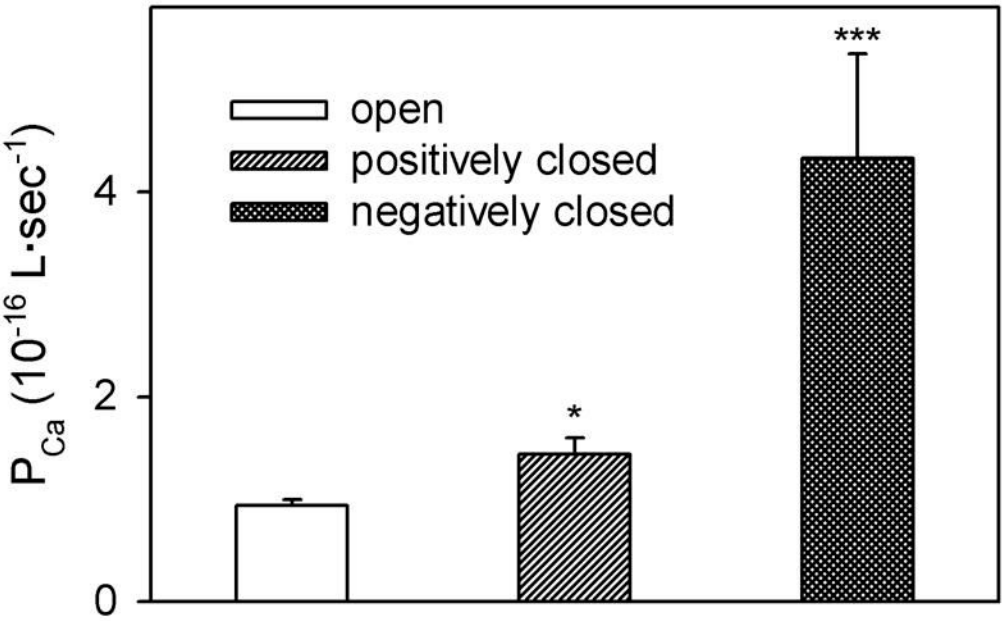

Fig. 4.

Comparison of the permeability of Ca^{2+} through VDAC in different states. The values are mean ± S.E. of 15 measurements in the open state and the negatively closed states and 19 measurements in the positively closed states. (*P < 0.05, *** P < 0.001, compared to the open state)

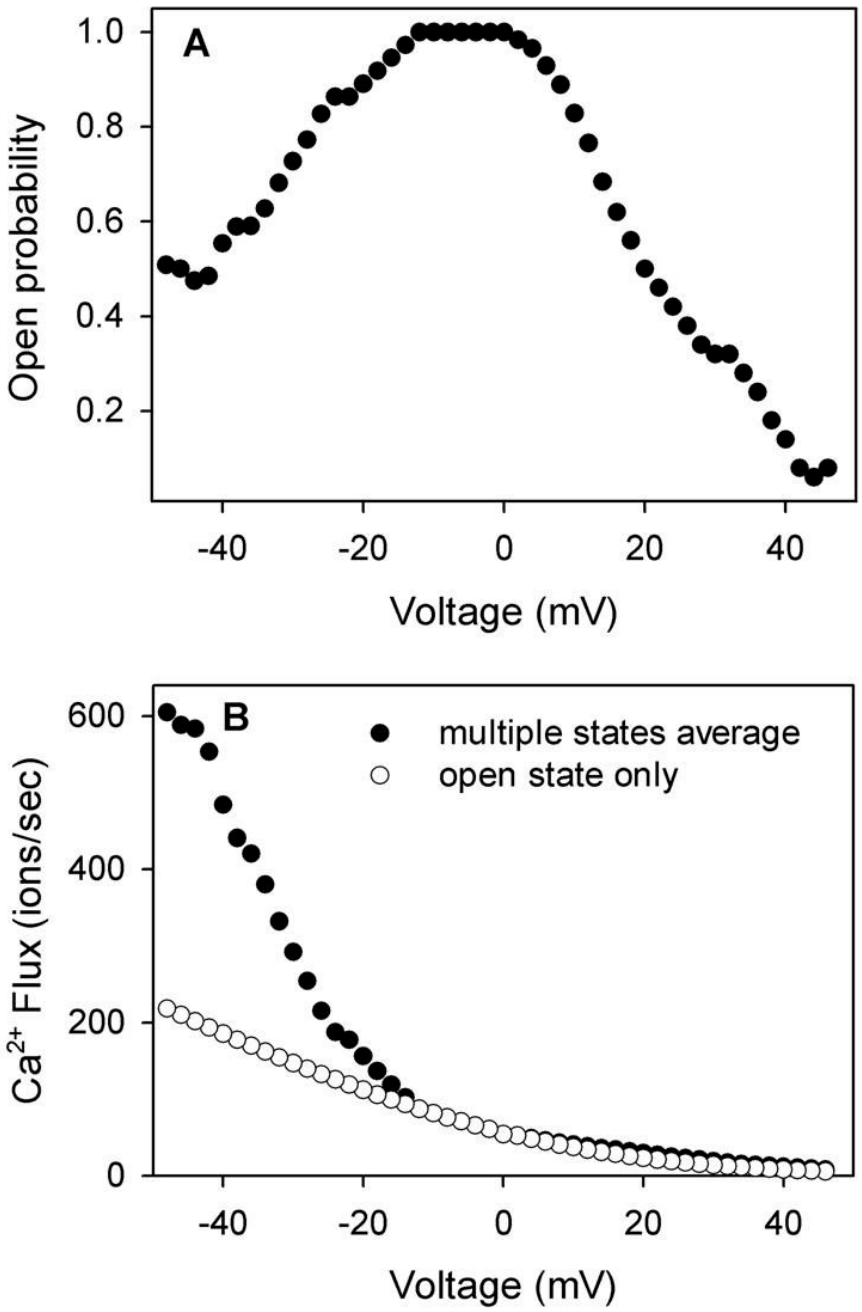

Fig. 5.

(A) The open probability of VDAC in response to voltage. **(B)** A comparison of the average calcium flux through a single VDAC channel in response to voltage calculated for what it would be in the presence of 1 μM cytosolic Ca^{2+} (0 μM in the intermembrane space). The filled circles are the weighted average flux of Ca^{2+} through the open and closed states, assuming the same voltage gating property as in panel A. The open circles are assuming that VDAC is always in the open state.

Table 1

Comparison of the conditions and results

	This paper	**Gincel *et al.* [6]**
$CaCl_2$ gradient	80 mM: 20 mM	250 mM: 150 mM
Ionic strength	150 mM	600 mM
Debye length	0.78 nm	0.39 nm
P_{Ca}/P_{Cl}	0.04	0.35