J. Membrane Biol. 123, 83–91 (1991)
002226319100110F

The Journal of
Membrane Biology

Patch Clamping VDAC in Liposomes Containing Whole Mitochondrial Membranes

Uwe R. Wunder* and Marco Colombini
Laboratories of Cell Biology, Department of Zoology, University of Maryland, College Park, Maryland 20742

Summary. Whole mitochondrial membranes isolated from *Neurospora crassa* were reconstituted into liposomes and patch clamped. Clear activity characteristic of the mitochondrial channel VDAC was found, namely: open state conductance of 650 pS (in 150 mM KCl, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.2), voltage-dependent closure at both positive and negative potentials, change in conductance upon channel closure of about 450 pS in response to negative and positive potentials, and increased voltage dependence in the presence of König's polyanion. This is the first clear demonstration of VDAC single channels using the patch-clamp technique, even though others used this method before to study whole mitochondrial membranes and liposomes containing mitochondrial proteins. We also found one other channel with a conductance change of about 120 pS.

Key Words ion channels · mitochondrial membranes · voltage dependence · VDAC · *Neurospora crassa*

Introduction

Ionic channels are a special class of membrane proteins that allow passive transport processes to occur through aqueous pores. They are regulated by such different means as ligand binding, mechanical stretch, or a transmembrane voltage. Voltage-dependent channels are important in many physiological processes such as muscle movement, orientation in bacteria, and fertilization (Jan & Jan, 1989). They are also found in a variety of organelles, e.g. synaptic vesicles (Thomas et al., 1988), endoplasmic reticulum (Schmid et al., 1988), sarcoplasmic reticulum (Holmberg & Williams, 1990), nuclear envelope (Mazzanti et al., 1990), and in mitochondria (Colombini, 1979; *cf. also* the Table).

The first discovered (Schein, Colombini & Finkelstein, 1976) and best characterized intracellular channel is VDAC (voltage-dependent anion-selective channel) of the mitochondrial outer membrane (Colombini, 1989; Dihanich, 1990). It is probably the major pathway between the cytosol and the mitochondrial intermembrane space. VDAC's electrophysiological properties were studied extensively in planar phospholipid membranes. It is sensitive to transmembrane potentials and responds symmetrically to positive and negative voltages. VDAC has a highly conducting open state of 4 to 4.5 nS in 1 M KCl (equivalent to about 700 pS in 150 mM KCl, since VDAC's conductance in KCl is linear with salt activity) and less conducting ("closed") states whose conductance averages at about 40% of that of the open state (an expected value of about 300 pS in 150 mM KCl) (Colombini, 1986). Its presumably simple beta-sheet conformation (Blachly-Dyson et al., 1990) makes VDAC an ideal model protein for basic research on protein dynamics and function (Mannella & Tedeschi, 1987).

Recently the patch-clamp method was introduced to study the electrophysiological properties of mitochondrial membranes (Sorgato, Keller & Stühmer, 1987). A host of different ion channels have been reported in the inner and outer mitochondrial membrane (*cf.* Table). But, despite the many experiments done so far, clear VDAC activity was not found. This led Fèvre et al. (1990) to believe that their experimental protocol inactivated VDAC. Sorgato et al. (1987) attributed a 350-pS conductance change to VDAC, but prefer to leave the question open in the light of their most recent experiments (Moran et al., 1990).

We found it surprising that it should not be possible to detect VDAC with the patch-clamp method. VDAC makes up as much as half the protein content of the outer mitochondrial membrane (Mannella, 1982), and it is unlikely that all the protein is denatured in the preparation. We also could not think of any reason inherent in the patch-clamp method that would prevent the detection of VDAC. Thus we decided to patch clamp liposomes (Tank & Miller,

* *Current address:* Abteilung Biochemie, Universität Witten/Herdecke, Stockumer Str. 10, D-5810 Witten, Germany.

1983) that contained reconstituted whole mitochondrial membranes. We expected to find VDAC, but also a variety of other conductances as indicated in the literature.

The results reported here suggest that VDAC is the major mitochondrial channel that can be detected with a slow time response. The voltage dependence, open and closed state conductance, and sensitivity to König's polyanion (Colombini et al., 1987) are consistent with VDAC being present in most of our patches. One channel with a behavior that can be attributed to the 107-pS channel (Sorgato et al., 1987) was also seen.

Materials and Methods

Preparation of the Phospholipid/Protein Powder

Whole mitochondrial membranes of *Neurospora crassa* at a final concentration of 0.66 mg protein/ml buffer A (1 mM KCl, 1 mM 4-(2-hydroxyethyl)-piperazineethanesulfonic acid (HEPES) (sodium salt, pH 7.0), 15% vol/vol dimethylsulfoxide (DMSO)) were purified after Colombini (1980). One ml of this suspension was diluted in 10 ml buffer B (1 mM KCl, 1 mM HEPES (pH 7.0)) and centrifuged for 20 min at 10,000 × *g* to remove the DMSO. The pellet was resuspended in 1 ml buffer B (giving solution 1). Then 18.4 mg phosphatidylcholine (Avanti 810051) and 1.6 mg phosphatidic acid (Avanti 840101) were dried down with nitrogen, resuspended in 1 ml buffer B and sonicated (Solution 2). Solutions 1 and 2 were mixed, sonicated, and distributed over five microfuge vials (i.e., 400 μl per vial). Various dilutions of this mixture with 20 mg soybean phospholipids (purified as in Kagawa & Racker, 1971) per ml of buffer B were made and lyophilized. A 1 : 7 dilution (5 μl of the mixture plus 35 μl soybean-phospholipids solution per vial) gave channel activity and was used throughout the experiments. Lower dilutions yielded no good seals while higher dilutions resulted in less activity.

Patch-Clamp Glass

Capillary tubes (Garner glass 7052, 1.1 mm inner diameter, 1.5 mm outer diameter) were pulled with the DKI C700 vertical puller equipped with a Chromel A wire by the two-stage process basically described in Hamill et al. (1981). The open electrodes had resistances of 1–10 MΩ in 150 mM KCl buffered solution and tip diameters between 1–4 μm. The electrodes were neither polished nor coated.

Experimental Conditions

Big multiwalled liposomes (up to 100 μm) were made by resuspending one vial of the lyophilisate of the 1 : 7 dilution with gentle swirling in 100 μl buffer C (made of 150 mM KCl, 20 mM HEPES (sodium salt, pH 7.2), 1 mM $CaCl_2$, passed through a 0.45 μm filter). One μl of this suspension was put onto a petri dish of 3 cm diameter and diluted with 2 ml of buffer C. As seen under the microscope (600 ×), some of the liposomes adhered to the surface of the plastic dish. The electrode (filled with buffer C) was lowered to a liposome under positive pressure and gently pressed against it. Slight suction resulted in an increase in resistance from 1–10 to 200–1000 MΩ (sometimes seals formed spontaneously). Seal formation was monitored with an oscilloscope (Nicolet 310) while applying rectangular voltage pulses. Then the electrode was pulled away from the liposome. In about half of the experiments the electrode was exposed to air for about 2 sec. Every second try gave a tight seal, and 90% of the membranes showed channel activity with 1–6 channels per patch. In the experiments with the modulator the electrode contained an additional 5 μg/ml König's polyanion in buffer C (Colombini et al., 1987). Patches lasted for up to 20 min. All experiments were performed at room temperature.

Electronics

Triangular and rectangular waves were applied with a Wavetek (model 184) function generator. The patch was voltage clamped using an operational amplifier (52K from Analog Devices) in the inverted mode. A 1 GΩ feedback resistor was used. The Ag-AgCl electrode in the patch pipette was connected to the negative input of the amplifier and thus was held at virtual ground. The command voltage was applied to the Ag-AgCl electrode in the bath. The output from the amplifier was recorded with a chart recorder (Kipp & Zonen BD41) with a pen response time of 0.4 sec for 0 to 95% full scale deflection. All reported voltages refer to the bath whose potential was controlled relative to the potential within the pipette.

Analysis of the Data

The conductance at any time in a membrane containing one or a few channels was analyzed manually by determining the slope of the records generated with a triangular wave (*see* Fig. 2). Only events that decreased and subsequently increased the conductance were analyzed (except for channel insertions and exsertions, *cf.* Results). The conductance (G) equals the change in current (dI) per time interval (dt) divided by the change in voltage (dV) per time interval:

$$G = dI/dt \cdot dt/dV. \tag{1}$$

The analysis of the voltage dependence is as described by Schein et al. (1976). A 5 mHz triangular voltage wave was applied to the membranes and the resulting current recorded. The recordings were digitized and converted to conductance values by dividing the current at any time by the corresponding voltage value. Only that part of the wave during which the electric field was decreasing with time was used for subsequent analysis, since channel opening of VDAC is much faster than channel closure.

Assuming a two-state process, the conductance : voltage curves were fitted to the Boltzmann distribution as follows:

$$\ln[(G_{max} - G)/(G - G_{min})] = (nFV - nFV_0)/RT. \tag{2}$$

G, G_{max}, and G_{min} are the conductance at any voltage V, the maximum conductance (all channels open), and the minimum conductance (all channels closed), respectively. F, R, and T are the Faraday constant, the gas constant, and the absolute temperature, respectively. V_0 is the voltage at which one-half of the

channels are closed. The value of n is a measure of the steepness of the voltage dependence. $(G_{max} - G)/(G - G_{min})$ is equal to the ratio of the number of closed to the number of open channels. Plots of $\ln[(G_{max} - G)/(G - G_{min})]$ *vs.* V yielded n and V_0 (*see* Fig. 4).

Results

Whole mitochondrial membranes reconstituted into liposomes were patch clamped. Every second patch gave a tight seal, and 90% of the membranes contained 1–6 channels. Clear channel activity was found. We found, as did Moran et al. (1990), that it does not make a detectable difference to measure in the attached or in the excised patch-clamp mode. This is presumably due to the liposome membrane containing so many channels that it does not contribute a significant series resistance. Only once was an increase in the conductance of the patch observed after air exposure. The change in conductance of this membrane was 290 pS in the 150 mM KCl solution (buffer C, *see* Materials and Methods).

Figure 1 shows a record of current flowing through a membrane patch (top trace) in response to different applied potentials (lower trace). At elevated positive potentials discrete current drops were recorded and interpreted as channel closings events. The channels closed at relatively slow rates (many seconds), but when the voltage was returned to zero the channels reopened rapidly. The reopening was detected by the elevated current following the immediate reapplication of a membrane potential. Fast opening and slow closure are a characteristic of VDAC channels. In this experiment, the channels did not close readily at negative command voltages.

Typically, after 5–10 min the channels appeared to leave the patch (perhaps by diffusing into the glass-membrane seal region). The right side of Fig. 1 and the inset show the loss of a channel in two ways: (i) as a slow, continuous reduction in current and (ii) as a discrete drop in current. Once a channel was lost, it did not return.

The presence of voltage-gated channels in these patches is also demonstrated in Fig. 2. Here the voltage was changed continuously with time in the form of a slow (5 mHz) triangular wave. Thus the current should also have changed in a triangular fashion except when changes in conductance occurred. In the positive voltage region (right side of figure) channel closures (downward deflections) occurred as the voltage was rising while openings (upward deflections) were observed as the voltage declined. Approximately five channels closed and reopened in a narrow voltage region, indicative of steep voltage dependence. As in the case of VDAC,

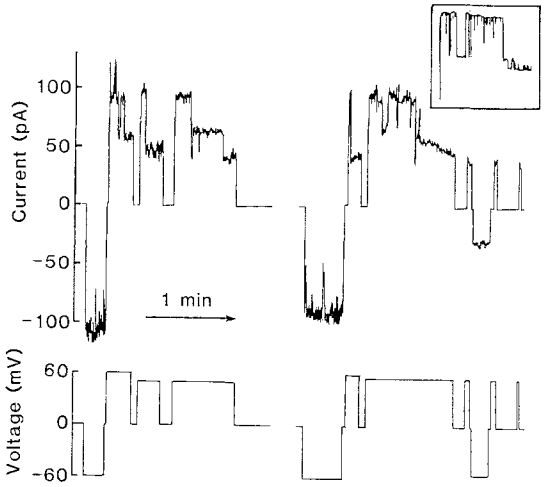

Fig. 1. Current record in a patch containing two channels at the beginning. The recording was made on liposomes containing whole mitochondrial membranes by using the patch-clamp method as described in Materials and Methods. The upper tracings are the current records in response to the applied voltages shown below. The solution contained 150 mM KCl, 20 mM HEPES (sodium salt, pH 7.2), 1 mM $CaCl_2$, 0.45 μm filtered. The sign of the applied potential refers to the bath. There is a 3-min gap in the middle of the record. Time progressed in the direction indicated by the 1-min long time arrow. The inset presents a recording made on a different patch of membrane under the same conditions. The voltage-induced conductance changes with positive potentials for the left side of the chart were: 0.49 and 0.58 nS for the upper and lower conductance levels in the first pulse; 1.0 nS for the second pulse (probable simultaneous closure); and 0.47, 0.51, 0.47, and 0.47 nS for the third pulse. Similarly for the right side, the conductance changes were: 1.02 nS for the first pulse; 0.13, 0.45, 0.57, and 0.57 nS for the second. The nondiscrete conductance drop toward the end of the second positive potential pulse on the right side resulted in a total conductance decrement of 0.27 nS. In the inset, the conductance changes were 0.47, 0.47, 0.45, and 0.10 nS followed by a slow baseline decrement of 0.03 nS (the applied voltage was 55 mV)

slow closing kinetics resulted in significant hysteresis, i.e., channels closed at higher voltages and reopened at lower voltages. As was the case for the experiment illustrated in Fig. 1, channels closed more readily at positive potentials.

Figure 3 shows a histogram of 185 conductance changes observed when triangular waves were applied to patches containing channels (as in Fig. 2). Only one broad peak around 450 pS is evident. The values are widespread from 80 to 680 pS. The nine largest events in the histogram (between 910 and 1350 pS) appeared to be single events but may also have resulted from unresolved multiple events.

In order to compare the voltage dependence of the channels observed in the patches to that reported for VDAC channels reconstituted in planar phospholipid membranes, the voltage-dependence parame-

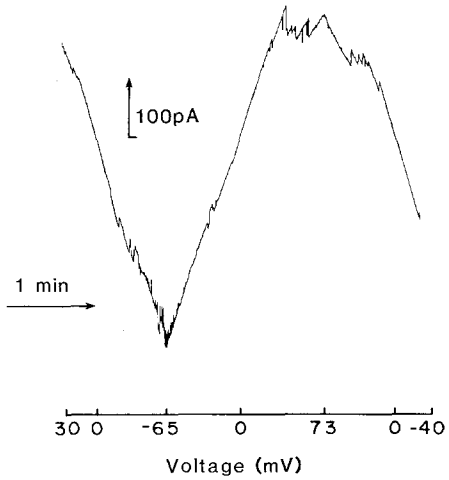

Fig. 2. Current record, in a patch containing at least five channels, in response to a triangular voltage wave. Experimental conditions were as in Fig. 1. The applied voltage was changed linearly with time in the form of a 5-mHz triangular wave. The voltage axis at the bottom shows the peak voltages of the triangular wave and the beginning and ending voltage for the current record illustrated. The base of the current vector shows the location of zero current

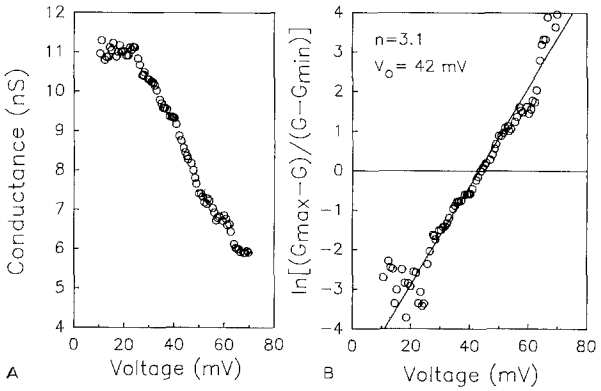

Fig. 4. (*A*) Conductance to voltage relation for channels in one membrane patch. Six records were averaged to obtain the results illustrate in the figure. Experimental conditions were as in Fig. 1. (*B*) The data plotted in *A* were fitted to a Boltzmann distribution as described in Materials and Methods. At the point where the data crosses zero on the *y* axis, the voltage corresponds to V_0 and the number of open channels equals the number of closed channels. The parameter n is the slope divided by F/RT and estimates the steepness of the voltage dependence (*cf.* Materials and Methods). The straight line was generated by the method of least squares

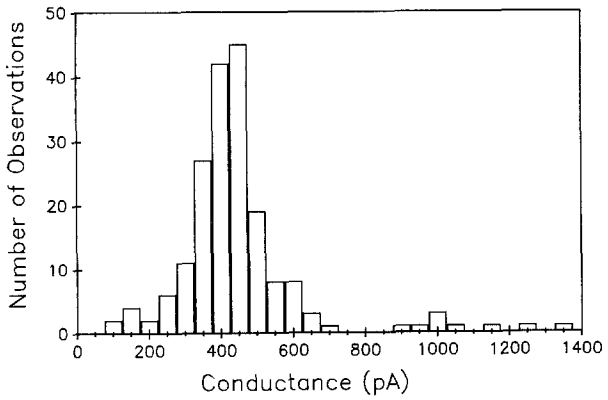

Fig. 3. Histogram of the changes in conductance obtained from records such as the one illustrated in Fig. 2. Only one cycle of the triangular wave was included per recording

ters were quantitated using records such as the one illustrated in Fig. 2. The records were digitized and conductances were calculated as a function of voltage. In order to reduce the fluctuations inherent in records containing few channels, records made on the same patch were often averaged. Figure 4*A* shows an averaged conductance : voltage plot. The conductance of the membrane decreased with increasing voltage as more and more channels closed. Figure 4*B* shows the same data fitted to a two-state model (*see* Materials and Methods). In this experiment, half the channels closed at 42 mV, i.e., the parameter $V_0 = 42$ mV. The parameter n that quantitates the steepness of the voltage dependence was 3.1. Averaging the values obtained by analyzing data recorded from different patches yielded the following: At negative applied potentials $V_0 = 29 \pm 5$ mV (SD) and $n = 4.4 \pm 0.9$ (SD) for six patches; at positive applied potentials $V_0 = 38 \pm 6$ mV (SD) and $n = 3.9 \pm 0.9$ (SD) for seven patches.

We often observed an asymmetric voltage dependence in the sense that the channels closed less frequently at negative command voltages. When the channels closed at negative potentials, it was at a higher voltage (in the absolute sense) than at positive potentials. In addition, the reopening with decreasing negative potentials frequently occurred at lower voltages (closer to zero) as compared to the behavior at positive potentials.

Most of our records show voltage-dependent channel behavior for no longer than 15 min. Figure 5 illustrates two occasions in which channel loss was recorded. In the bottom trace, the channel opens up before it is lost in its open state. The loss of conductance resulted in only a small downward deflection because the event occurred at a low potential. The dramatic decrease in slope, corresponding to a conductance change of 650 pS, indicates the loss of the channel. In the upper trace, the channel seems to have been lost in the closed state, since no reopening event was observed. The channel loss was not associated with an abrupt current change because it occurred close to 0 mV. The conductance changed by 470 pS as evidenced by the change in

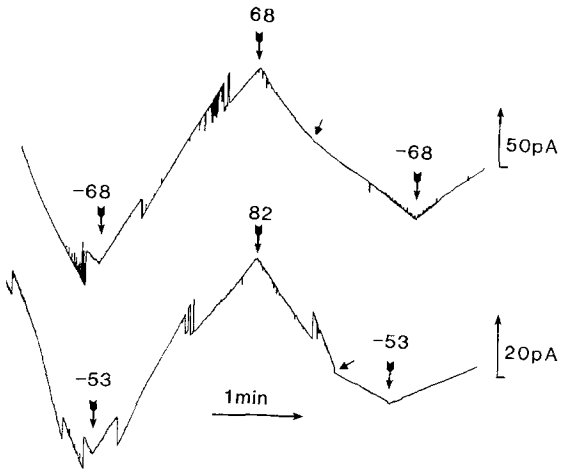

Fig. 5. Two examples of channel loss from the patch. Experimental conditions were as in Fig. 2. Triangular voltage waves (5 mHz) were applied, and arrows indicate the locations and value of the peak voltages of these waves. In the upper trace a channel was lost (small arrow) while in its closed state. No discrete change in the current can be seen since the event occurred close to 0 mV, but a change in the slope occurred. In the lower trace (from a different patch) a channel responding symmetrically to voltage was lost (small arrow) while in the open state

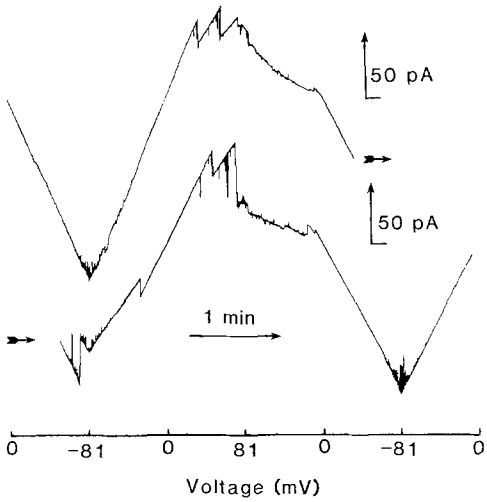

Fig. 6. The effect of König's polyanion on the channels. In this experiment the electrode contained in addition to the normal salt solution (*cf.* Fig. 1) 5 μg/ml König's polyanion. Triangular voltage waves were applied with voltages as indicated in the scale at the bottom. The figure shows two successive cycles (indicated by horizontal arrows). Note the small conductance changes in the second cycle, while the channels are closed

the slope of the record while going from +68 to −68 mV.

In order to obtain more evidence to support our contention that the channels in the patch are indeed VDAC, we used König's polyanion, a substance known to increase VDAC's voltage dependence (Colombini et al., 1987). The polyanion is expected to increase the steepness of the voltage dependence and cause the channels to close at lower potentials. It is also known that polyanions act asymmetrically, the voltage dependence is increased if the polyanion-containing side is made negative (Mangan & Colombini, 1987). Thus König's polyanion was added either to the pipette solution or to the bath. In general, we found an increased voltage dependence with the polyanion that is characteristic for VDAC (Fig. 6). The channels tended to reopen at much lower voltages and the steepness was somewhat increased. In the figure, the channels opened very close to 0 mV, resulting in very small current transitions, but the increase in slope is evident. In the presence of polyanion in the bath, the channels closed at negative potentials in contrast to observations made in the absence of the polyanion (e.g., Fig. 2) or with the polyanion present in the pipette.

No clear effects were observed resulting from the application of suction to the patch. With voltage-gated channels present in the membrane, as much as 50 mm Hg of suction was applied. No reproducible shifts in the properties of the channels were found (*data not shown*). Since we did not determine the curvature of the patches on which suction had been applied, the actual tension applied to the membrane is not known.

Two of our recordings differed from all the others in that the conductance increased with increasing voltage. A 120-pS channel seemed to open up at positive potentials (Fig. 7). At negative potentials, little if any activity was observed over the expected increase in the noise. But when positive potentials are applied, the current increased and oscillated between two conductance levels separated by about 120 pS. This behavior was only seen twice in about 200 recordings. It is different from the other patches where voltages higher than ±25 mV resulted in a decrease in current.

Discussion

The recording of the electrical characteristics of single channels in a patch of membrane attached to the tip of a glass electrode (i.e., patch clamping) has proved to be a very powerful tool for studying channels present in a variety of membranes. For 15 years, the mitochondrial channel called VDAC has been isolated and reconstituted into planar phospholipid membranes. However, recent attempts to detect single channels with the characteristics of VDAC by the patch-clamp technique failed (e.g., Fèvre et al.,

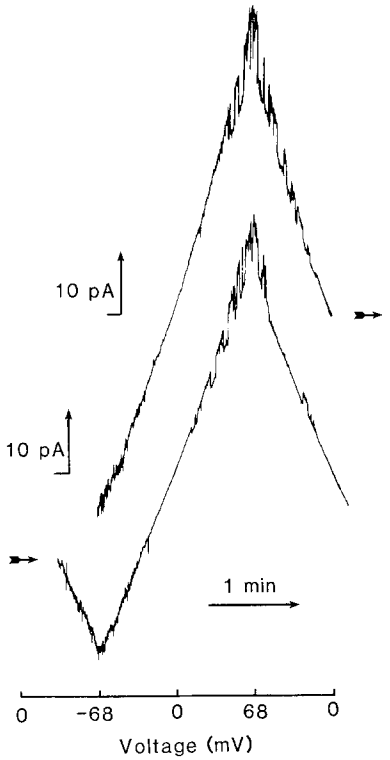

Fig. 7. Two successive cycles (indicated by arrows) of a recording with events of about 120 pS. Triangular voltage waves were applied to the patch with voltages as indicated in the scale at the bottom. Experimental conditions were as in Fig. 2

1990; Moran et al., 1990; Catia Sorgato, *personal communication*), even though VDAC's presence in the preparation could be shown by using an immunoblot (Catia Sorgato, *personal communication*).

By contrast, we found characteristic VDAC behavior in most of our measurements. We observed a voltage-dependent change in conductance from an open to a less conducting "closed" state with increasing membrane potential. The location of the switching region (V_0), around 30 mV, compares favorably with VDAC (Colombini, 1989). The ability of individual channels to close at both positive and negative potentials is also a characteristic of VDAC. The magnitude of the conductance change upon channel closure (Fig. 3) and its variability are both qualities possessed by VDAC. The slow rate of channel closure and its fast opening rate also correspond with what would be expected for VDAC channels. The few estimates of open channel conductance, when corrected for KCl activity, are also consistent with VDAC. Finally, König's polyanion increased the voltage dependence of the observed channels as it does for VDAC (Colombini et al., 1987).

Conductance changes corresponding to those reported here for VDAC's closure (*see* Fig. 3) were found by Moran et al. (1990). However, since these investigators did not observe an associated voltage dependence, they did not attribute these changes to the presence of VDAC. Thieffry et al. (1988) assumed that denaturation during the preparation of the mitochondrial membranes was responsible for VDAC not being observed in their experiments. Considering the slow rate of VDAC closure and the short time scale examined by these investigators, it is possible that they missed these changes.

The histogram (Fig. 3) shows nine values between 910–1350 pS that appeared to be single events. Since we often saw channels responding almost simultaneously we attribute these events to being unresolved multiple events. However, it must be noted that Zoratti, Petronilli and Szabò (1990) reported a 1300-pS channel and Moran et al. (1990) describe conductances around 1000 pS in mitochondrial membranes. Thus we cannot exclude the possibility that some of the events we observed could be other types of channels.

The origin of some of the low-conductance transitions (80–200 pS) observed could be channels originating from the inner (Kinally, Campo & Tedeschi, 1989) or the outer membrane (Moran et al., 1990; Catia Sorgato, *personal communication*). We indeed found sometimes very small events (e.g. in Fig. 6). Small conducting units could be a reason for the histogram ranging over such a broad interval.

An important characteristic of VDAC is its ability to close at both positive and negative transmembrane potentials. Figure 5 (upper trace) shows a record where the channel is closing at positive and negative potentials. The loss of the conductance that responds to positive potentials is always linked to the loss of that responding at negative potentials, indicating that they are due to the same conducting entity. In addition, the total decrement in conductance is consistent with the loss of only one channel. These findings indicate that one channel responded to both positive and negative potentials rather than two channels oriented in opposite directions.

When the voltage dependence was quantitated by fitting to a two-state system, the values of the voltage-dependent parameters are quite similar to those reported for VDAC (Colombini, 1989). The V_0 values are slightly higher (29 and 38 mV for negative and positive voltages, respectively, as opposed to 20 mV), but this is not uncommon. Such a change could result from a very small change in conformational energy difference between the open and closed states and can arise by simply changing the phospholipids in the membrane.

A more interesting observation is the difference in V_0 values that depend on the sign of the applied

Table. Single-channel recordings of mitochondrial channels using the patch-clamp technique

Author(s)	Method	Change in conductance (substates) in 150 mM salt (pS)	Selectivity	Voltage dependent	Membranes used			Species	Remarks
					Inner	Outer	Whole		
Fèvre et al., 1990	Tip-dip	330 (100, 140, and 240)	Cationic	Yes			X	Yeast	Proposed as an outer membrane channel
Thieffry et al., 1988	Tip-dip	220 (100)	Cationic	Yes			X	Rat brain	Proposed as an outer membrane channel
Moran et al., 1990	Patch-clamp on liposomes	475	Not reported	No			X	Rat brain	Preparation enriched in contact sites
		550		No			X		
		<100		No			X		
		250		Yes		X			
		1000		No			X		
Sorgato et al., 1987	Patch-clamp on mitoplasts	107	Anionic	Yes	X			Mouse liver	
Kinnally et al., 1989	Patch-clamp on mitoplasts	45	Anionic	No	X			Mouse liver	
		120–150	Cationic	Yes	X				
		350	Cationic	Yes	X				
		1000	Not reported	Yes	X				
Zoratti et al., 1990	Patch-clamp on mitoplasts	1300 (many)	Not reported	Yes			X	Rat liver	Proposed as inner membrane channel

potential. These differences can be much more dramatic than indicated in the Results because, if the channels closed very poorly or not at all, they could not be analyzed. Although also observed in bilayer experiments, this phenomenon was very consistent in that channels closed less readily when negative potentials were applied. However, when closure occurred, the voltage dependence of opening tended to be greater (i.e., n was greater). This more pronounced hysteresis may be due to the curvature of the membrane patch, which is likely not to occur in the planar bilayers. Since evidence indicates that VDAC carries a positively-charged sensor (Bowen, Tam & Colombini, 1985; Doring & Colombini, 1985; Mirzabekov & Ermishkin, 1989), a negative potential would move the sensor toward a compressed membrane monolayer. This added structural constraint may explain the decreased tendency to close and the delay in opening (the lower V_0 may be a kinetic delay). Positive potentials would drive the sensor to the monolayer under local tension. This probably provides less steric hindrance, resulting in more likely closure.

One problem with the patch-clamp method is that it can only distinguish changes in conductances. VDAC is a channel that does not close all the way when responding to a voltage, thus leaving a residual conductance. In the bilayer, VDAC usually inserts in the open state, allowing one to determine the entire open-state conductance. Voltage-dependent closure results in the loss of only part of the conductance. By difference, the closed-state conductance can be calculated. The size of the residual conductance cannot usually be determined with the patch-clamp technique. We overcame this drawback, at least in part, by examining channel losses. Figure 5 (upper trace) shows a channel that at first responded symmetrically to applied voltage but then was lost. The conductance change of 650 pS is consistent with VDAC (Colombini, 1989). After correcting for salt activity we expect about 700 pS.

König's polyanion is known to specifically increase VDAC's voltage-dependence (Colombini et al., 1987). We added 5 μg/ml to our normal solution and found that the channel under investigation was more sensitive to voltage (Fig. 6). Thus our results support the hypothesis that we really saw VDAC activity in our patches.

An advantage of the patch clamp as compared to reconstitution in planar membranes is the possibility to test channels for stretch sensitivity. Our attempts to find any clear stretch-induced changes in the voltage dependence of VDAC were not successful. However, since we did not measure the curvature of the patch, the actual tension applied may not have been sufficient.

The only channel clearly different from the recordings we attribute to VDAC had a conductance change of about 120 pS and was seen in two patches (Fig. 7). It behaved asymmetrically and its probability of being open increased with increasing voltage. It probably represents the channel of the inner mitochondrial membrane reported by Sorgato et al. (1987).

The Table summarizes some of the mitochondrial channels that have been proposed to exist by using the patch-clamp method. Most of the changes in conductance reported by other authors are present in our recordings (Fig. 3). Most of these can be attributed to VDAC because they are associated with its voltage-gating process, but we cannot exclude the possibility that some are due to channels superimposed on the VDAC conductance. It is also possible that we did not resolve other channels since we were looking at a slow time response. All other investigators used a higher time response. Based on our observations, we suggest that the large variety of conductance changes reported by different investigators may represent the manifestations of just a few channel-forming structures.

The issues raised by the recent reports on a variety of different channels in the inner and outer membrane of mitochondria (Table) still wait to be resolved. The physiological role of these channels remains unclear, especially in view of the presence of the other mitochondrial transport systems.

This work was supported by the Office of Naval Research (ONR) Grant N00014-90-J-1024 to M.C. and the German Academic Exchange Service (DAAD) for U.W.

References

Blachly-Dyson, E., Peng, S., Colombini, M., Forte, M. 1990. Selectivity changes in site-directed mutants of the VDAC ion channel: Structural implications. *Science* **247:**1233–1236

Bowen, K.A., Tam, K., Colombini, M. 1985. Evidence for titratable gating charges controlling the voltage dependence of the outer mitochondrial membrane channel, VDAC. *J. Membrane Biol.* **86:**51–59

Colombini, M. 1979. A candidate for the permeability pathway of the outer mitochondrial membrane. *Nature* **279:**643–645

Colombini, M. 1980. Pore size and properties of channels from mitochondria isolated from *Neurospora crassa*. *J. Membrane Biol.* **53:**79–84

Colombini, M. 1986. Voltage gating in VDAC: Toward a molecular mechanism. *In:* Ion Channel Reconstitution. C. Miller, editor. pp. 533–552. Plenum, New York

Colombini, M. 1989. Voltage gating in the mitochondrial channel, VDAC. *J. Membrane Biol.* **111:**103–111

Colombini, M., Yeung, C.L., Tung, J., König, T. 1987. The mitochondrial outer membrane channel, VDAC, is regulated by a synthetic polyanion. *Biochim. Biophys. Acta* **905:** 279–286

Dihanich, M. (editor) 1990. Multi-author review: Pore-forming proteins of biological membranes. *Experientia* **46:**131–201

Doring, C., Colombini, M. 1985. Voltage dependence and ion selectivity of the mitochondrial channel, VDAC, are modified by succinic anhydride. *J. Membrane Biol.* **83:**81–86

Fèvre, F., Chich, J.-F., Lauquin, G.J.M., Henry, J.-P., Thieffry, M. 1990. Comparison of mitochondrial cationic channels in wild-type and porin-deficient mutant yeast. *FEBS Lett.* **262:**201–204

Hammill, O.P., Marty, A., Meher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Holmberg, S.R.M., Williams, A.J. 1990. The cardiac sarcoplasmic reticulum calcium-release channel. *Biochim. Biophys. Acta* **1022:**187–193

Jan, L.Y., Jan, N.J. 1989. Voltage-sensitive ion channels. *Cell* **56:**13–25

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J. Biol. Chem.* **246:**5477–5487

Kinnally, K.W., Campo, M.L., Tedeschi, H. 1989. Mitochondrial channel activity studied by patch-clamping mitoplasts. *J. Bioenerg. Biomembr.* **21:**497–505

Mangan, P.S., Colombini, M. 1987. Ultrasteep voltage dependence in a membrane channel. *Proc. Natl. Acad. Sci. USA* **84:**4896–4900

Mannella, C.A. 1982. Structure of the outer mitochondrial membrane: Ordered arrays of pore-like subunits in outer-membrane fractions from *Neurospora crassa* mitochondria. *J. Cell Biol.* **94:**680–687

Mannella, C.A., Tedeschi, H. 1987. Importance of the mitochondrial outer membrane channel as a model biological channel. *J. Bioenerg. Biomembr.* **19:**305–308

Mazzanti, M., DeFelice, L.J., Cohen, J., Malter, H. 1990. Ion channels in the nuclear envelope. *Nature* **343:**764–767

Mirzabekov, T.A., Ermishkin, L.N. 1989. The gate of mitochondrial porin channel is controlled by a number of negative and positive charges. *FEBS Lett.* **249:**375–378

Moran, O., Sandri, G., Panfili, E., Stühmer, W., Sorgato, M.C. 1990. Electrophysiological characterization of contact sites in brain mitochondria. *J. Biol. Chem.* **265:**908–913

Schein, S.J., Colombini, M., Finkelstein, A. 1976. Reconstitution in planar lipid bilayers of a voltage-dependent anion-selective channel obtained from *Paramecium* mitochondria. *J. Membrane Biol.* **30:**99–120

Schmid, A., Gögelein, H., Kemmer, T.P., Schulz, I. 1988. Anion channels in giant liposomes made of endoplasmic reticulum vesicles from rat exocrine pancreas. *J. Membrane Biol.* **104:**275–282

Sorgato, M.C., Keller, B.U., Stühmer, W. 1987. Patch-clamping of the inner mitochondrial membrane reveals a voltage-dependent ion channel. *Nature* **330:**498–500

Tank, D.W., Miller, C. 1983. Patch-clamped liposomes. *In:* Single Channel Recording. B. Sakmann and E. Neher, editors. pp. 91–105. Plenum, New York

Thieffry, M., Chich, J.-F., Goldschmidt, D., Henry, J.-P. 1988. Incorporation in lipid bilayers of a large conductance cationic channel from mitochondrial membranes. *EMBO J.* **7:**1449–1454

Thomas, L., Hartung, K., Langosch, D., Rehm, H., Bamberg, E., Franke, W.W., Betz, H. 1988. Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. *Science* **242:**1050–1053

Zoratti, M., Petronilli, V., Szabò, I. 1990. The giant channel of the mitochondrial inner membrane. *Biophys. J.* **57:**391*a*

Received 31 January 1991; revised 28 March 1991