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Patch Clamping the Outer Mitochondrial Membrane

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Summary. Intact giant mitochondria isolated from the liver of mice fed a diet containing cuprizone were studied using patch microelectrodes. The current-voltage curves were nonlinear, suggesting the presence of voltage-sensitive channels. In the negative range of voltage, the channels appear to close with increasing magnitude of the voltage. The dependence of the conductance on voltage is similar to that of the outer membrane channels (VDAC) studied in planar bilayers. Occasionally, over a narrow range of positive potentials, the conductance also decreases as in the bilayer studies. However, more frequently the conductance increases sharply in a completely reversible manner at potentials greater than 10 to 20 mV. The increase in conductance with voltage may be interpreted as a major rearrangement of membrane components. Qualitatively comparable results were obtained using fused outer membranes isolated from *Neurospora* mitochondria.

The behavior of VDAC is affected by treatment with succinic anhydride or the polyanion, polymethacrylate, maleate, styrene (1 : 2 : 3). We have found similar effects in the negative range of potentials in patches from giant mitochondria treated in the same fashion.

Key Words mitochondrial outer membrane · channels · VDAC · porin

Introduction

Early studies have indicated a high permeability of the outer mitochondrial membrane to low molecular weight solutes. Apart from these findings, however, most of the information on the permeability properties of the outer membrane has been provided by indirect studies. A polypeptide, approximately 29,000 to 35,000 mol wt, has been isolated from outer mitochondrial membranes. When incorporated into bilayer membranes it forms voltage-sensitive channels, and in liposomes it has been shown to be size selective for a variety of compounds. (Schein, Colombini & Finkelstein, 1976; Colombini, 1979; Zalman, Nikaido & Kagawa, 1980; Freitag, Neuport & Benz, 1982; Linden, Gellerfors & Nelson, 1982; Mihara, Blobel & Sato, 1982; Roos, Benz & Brdiczka, 1982; Colombini, 1983).

Although these reconstitution studies have provided a good deal of information on the probable behavior of the outer membrane channels, very little is known about the permeability properties of the native membrane.

Patch-clamp techniques (Neher & Sakmann, 1976; Hamill et al., 1981) have introduced the study of portions of membranes in electrical or actual isolation. This approach has permitted the study of single channels in a variety of cells. To our knowledge this technique has not been previously used in the study of the mitochondrial membranes. In the present study, we used this technique to begin to fill gaps in information about the electrical characteristics of mitochondrial outer membranes.

The study has been carried out with giant mitochondria derived from the liver of mice maintained on a diet containing cuprizone (Bowman & Tedeschi, 1983) and with outer membrane vesicles isolated from mitochondria of the fungus *Neurospora crassa* (Mannella, 1982). The results confirm the presence of voltage-sensitive channels in both membranes. Assuming the same conductance as that of the channels studied with bilayers, our results are consistent with the presence of 100 to 1000 channels per patch. However, while the isolated and reconstituted channels close symmetrically with applied potentials, we generally observe closure with negative potentials only. In the positive range, there is a marked increase in conductance which is reproducible and reversible and therefore not likely to correspond to a breakdown phenomenon.

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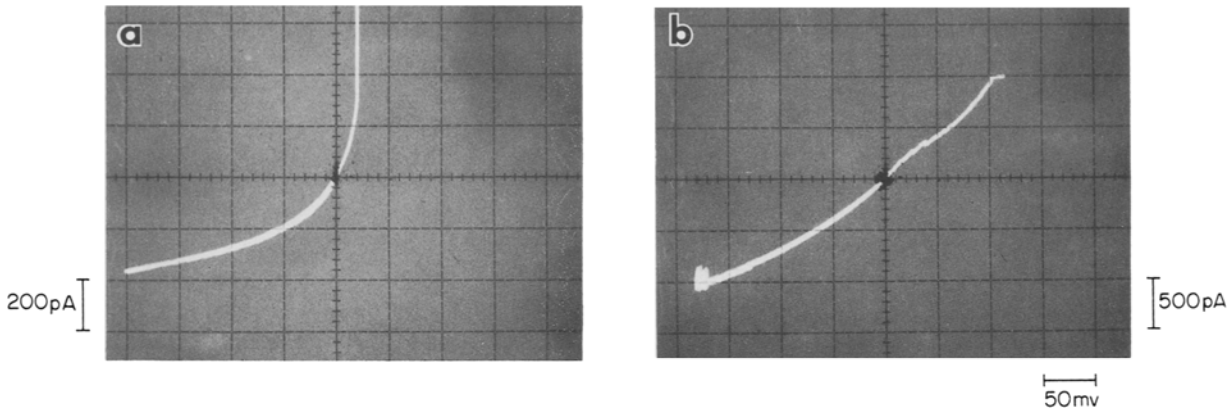

Fig. 1. Characteristic *IV* curves obtained with a giant mitochondrion using the electronic compensation for the in-series resistance (the micropipette resistance). (*a*) With this patch the conductance decreases with the magnitude of the potential in the negative range and increases in the positive range. This record is typical of those obtained. Figure 2 is a plot of conductance *vs.* voltage for this record. (*b*) This patch displays a decrease in conductance with that of voltage over the full range of negative potential and small positive potential with increase in conductance at higher positive voltages. This type of oscilloscope tracing was observed from 5 to 10% of the time. The discontinuities at the extremes of the record are electronic artifacts. Figure 3 is a plot of conductance *vs.* voltage for this record

Materials and Methods

Preparations

The giant liver mitochondria were isolated from mice of the Nya: NYLAR strain (Wadsworth Center for Laboratory Research, N.Y. State Department of Health, Griffin Laboratories, Guilderland, N.Y.), maintained on a diet containing cuprizone as previously described (Bowman & Tedeschi, 1983). Except in some of the early experiments, the proportion of cuprizone was reduced to 1.5 g per 500 g of standard rodent chow. The mitochondrial isolation procedure has been previously described (Bowman & Tedeschi, 1983). In the present study, only the bottom of 1 ml of the two-layer system was used for the final centrifugation. Unless otherwise specified, the mitochondria were suspended in 0.30 osmolal sucrose, 10 mM KCl and 5 mM HEPES (N-2-hydroxyethyl-piperazine-N′-ethane sulfonic acid), pH 7.0.

The outer membranes were isolated from *Neurospora crassa* mitochondria after hypoosmotic lysis and gradient centrifugation (Mannella, 1982). They were fused into large vesicles or sheets (some as large as 5 to 10 μm in diameter) (Schneider et al., 1980) by drying them overnight as a pellet in the presence of 5 mM potassium phosphate, pH 5.0, at room temperature.

Electronics

A Dagan 8900 patch clamp (Dagan Corporation, Minneapolis, MN) with an 8960 probe was used in conjunction with a 5111 Tektronix oscilloscope (Tektronix Inc., Beaverton, OR) equipped with two 5A19N amplifiers and a 5B10 time base amplifier.

In some recent experiments the electronics of the patch-clamp apparatus were modified following instructions from the Dagan Corporation to introduce a series resistance compensation of up to 300 MΩ.

Micropipettes

The patch-clamp pipettes were pulled and fire polished from capillaries 1 mm o.d. and containing an internal glass filament (IB100 F-4, World Precision Instruments, New Haven, CT), using a model PC-84 Sachs-Flaming micropipette puller (Sutter Instrument Co., San Rafael, CA). Typically, the pipette openings were 1.5 to 2 μm in diameter with a final segment 30 to 50 μm in length following the steep taper. The pipettes were filled with the medium used in the experiments except when otherwise specified. The resistance of the pipettes ranged from 100 to 300 MΩ when filled with 0.3 osmolal sucrose, 10 mM KCl, 5 mM HEPES, pH 7.0, and the current *vs.* voltage (*IV*) curve was linear, except in a few cases (approximately 1%) which were discarded. The PC-83 electrode holders of E.W. Wright (Gilford, CT) were used.

Patch Formation

A small volume of the mitochondrial or outer membrane vesicle suspension (generally 50 μl) was placed on glass sheets (35 × 60 mm and 0.13 to 0.17 mm in thickness) which had not been pretreated. The mitochondria were allowed to settle and attach to the glass and then generally washed with two 200-μl aliquots of the experimental medium and left in a final volume of approximately 200 μl. Only a small fraction of the mitochondria remained attached and with some preparations the washing had to be omitted. The mitochondria were viewed with a differential interference microscope with a total magnification of 480 or 1000× (*see* Bowman & Tedeschi, 1983). Deviations from these details are described in the legends of the figures.

Two general procedures were used. With the larger mitochondria (approximately 8 to 10 μm in diameter), the micropipette was advanced from above until its opening touched the mitochondrion, then suction was applied. More commonly, the pipette tip was advanced to within 2 to 5 μm of the vesicle, which was then attached to the tip by suction. Either technique was judged successful 10–20% of the time as judged from the change

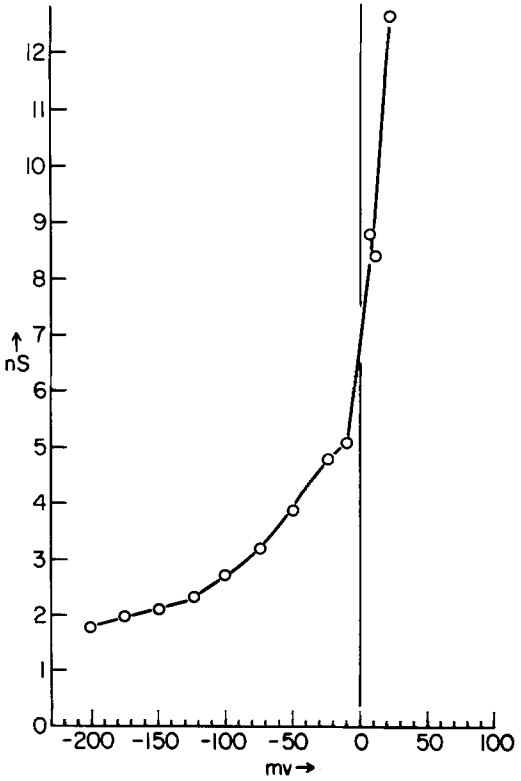

Fig. 2. Characteristic change in conductance with potential in a giant mitochondrion. The values were derived from the oscilloscope tracing of Fig. 1*a*

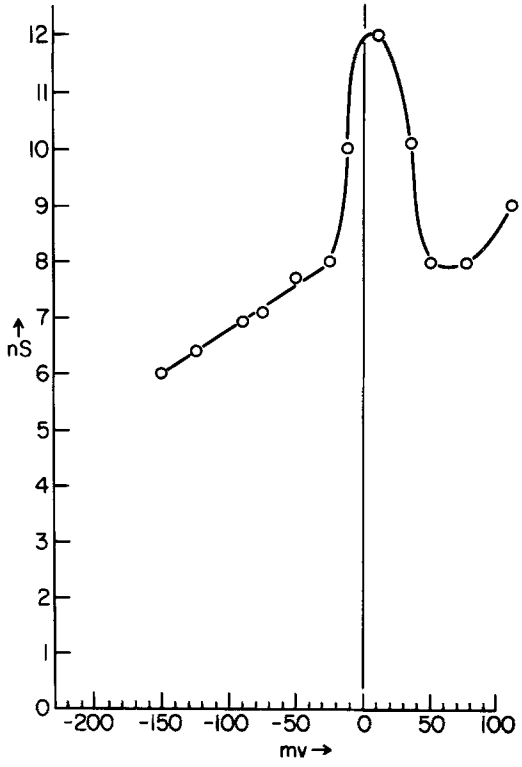

Fig. 3. Less commonly seen pattern of conductance as a function of potential in a giant mitochondrion. The values were derived from Fig. 1*b*

in electrical resistance and the characteristic *IV* curve. The first technique was used with the outer membrane preparations. Typically the patches ranged from 0.1 to 1 GΩ in resistance when bathed in solutions containing 10 mM KCl, 5 mM HEPES, pH 7.0, as measured with a positive test pulse of 20 mV.

The whole vesicles can remain attached to the pipette for minutes. Generally membrane material was taken up by the tip, sometimes in the case of mitochondria, as a cylinder as long as 2 μm. However, frequently only a small amount of material could be seen in the opening. Commonly, the vesicles would break away from the pipette without affecting the morphology or the electrical properties of the patch. The results were approximately the same regardless of configuration. The experiments were carried out at approximately 20°C.

The conductances were calculated from the oscilloscope records. The middle of the tracing of each *IV* curve was used. Where hysteresis was present either both curves were analyzed (e.g. Fig. 5) or the record showing the highest resistance was used (e.g. Fig. 2).

Results

Current-voltage (*IV*) curves were recorded directly as oscilloscope tracings in the voltage-clamp mode. The voltage was adjusted manually at a speed of approximately 0.2 to 0.5 sec/mV; the magnitude of the voltage was brought from 0 to −200 mV and then returned to 0. Generally the return curve exhibited higher resistances; i.e., the *IV* curves showed some hysteresis. The same procedure was followed in the positive range; however, generally we did not record above 10 to 100 mV (*see below*). In the latter range of potential the return record exhibited a decrease in resistance, similar in magnitude to the hysteresis observed in the negative range.

In initial experiments, the *IV* curve includes the pipette which acts as an in-series resistor. In these cases the voltage across the membranes was calculated from the data. In later experiments, the *IV* curves were corrected for the in-series resistance electronically and typical records obtained with the giant mitochondria are shown in Fig. 1*a* and *b*. When the results from the two different methods expressed as conductances were compared, they agreed within 10% and usually within 5%.

Figures 2 and 3 express the data of the *IV* curves of Fig. 1*a* and *b*, respectively, as conductances. Similar curves were obtained with outer membrane preparations as shown in Figs. 4–6. With either preparation the conductance was voltage de-

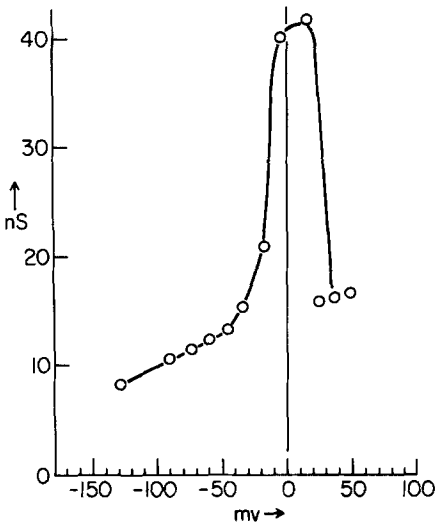

Fig. 4. Conductance as a function of potential for an outer membrane vesicle isolated from fungal mitochondria. The decrease in conductance with increased magnitude of the voltage was obtained in 1 out of 10 cases

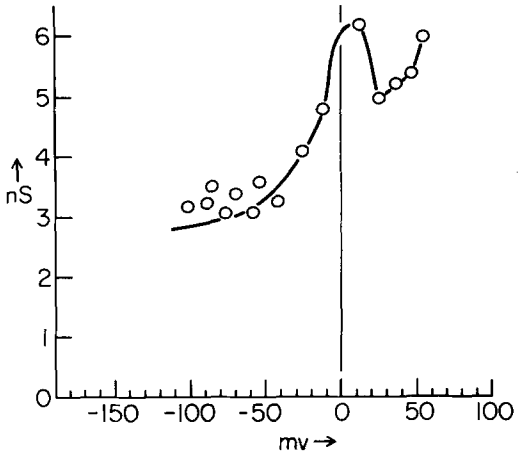

Fig. 5. Conductance as a function of potential for a fungal outer mitochondrial membrane vesicle. The pattern of decrease in conductance with increased magnitude of the voltage. Biphasic behavior in the positive range appeared in 3 out of 10 determinations

pendent. Figures 3, 4 and 5 are qualitatively similar (except at large positive voltage) to those published for the voltage-dependent anion-selective channels (VDAC) incorporated into bilayers after extraction from the mitochondrial outer membrane. The bilayer system exhibits a decrease in conductance with the magnitude of the potential, with 50% decrease occurring usually at transmembrane potentials of 40 to 50 mV (Bowen, Tam & Colombini, 1985). Generally, in our preparation, the decrease in conductance was observed in the same range for negative potentials. However, any decrease in conductance in the positive range of potential was either limited in range, or entirely absent, largely obscured or replaced by an increase in conductance not previously described for reconstituted VDAC.

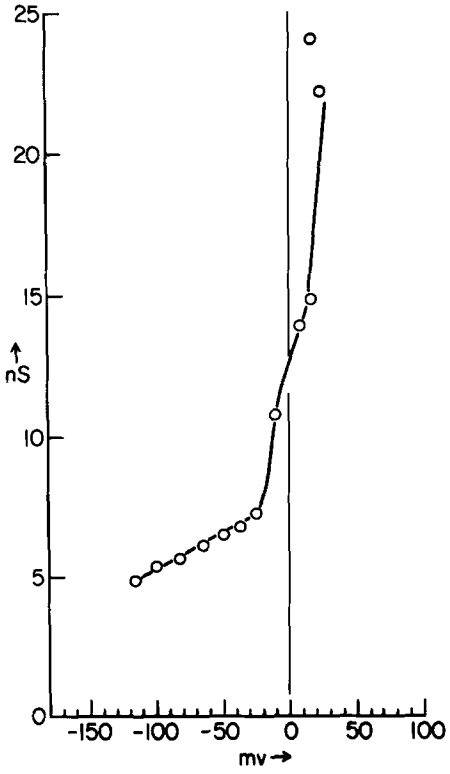

Fig. 6. Conductance as a function of potential for a fungal outer mitochondrial membrane vesicle. The pattern of increase in conductance with increase in potential in the positive voltage range appeared in 6 out of 10 cases

The closing of channels in the negative range of potentials may be explained by the presence of the VDAC, and those results pertinent to this question will be presented first.

Yeung et al. (1986) have shown that a synthetic polyanion (1 : 2 : 3 methacrylate, maleate, styrene), which interferes with a variety of mitochondrial functions (Konig et al., 1977), reduces the conductance of bilayers containing VDAC.

The addition of 42 μM of the polyanion to the mitochondria reduced the conductance measured with negative potentials. Representative *IV* curves are shown in Fig. 7 corresponding to records in the presence (*a*) and in the absence (*b*) of the polyanion. The times indicated for each oscilloscope tracing correspond to the times at which the record was taken and to those shown in Fig. 8. The position of the beam was adjusted manually to include all records in the same photograph. As shown, the records are in chronological order with the earlier traces underneath the later traces in the negative

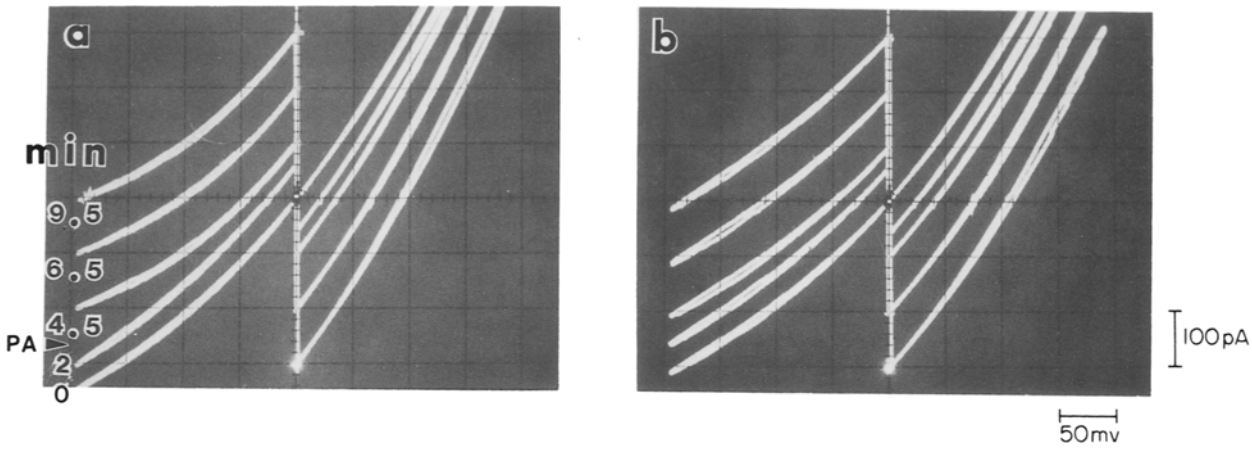

Fig. 7. The effect of the polyanion on *IV* curves of a giant mitochondrion. No series resistance compensation. At zero potential, the origin was shifted manually upwards for the negative potentials and downward for the positive potentials to trace several *IV* curves in the same record. (*a*) Addition of 42 μM polyanion (final concentration). (*b*) Controls

Table. Effect of the polyanion on conductance in the negative range of voltage[a]

Experiment	Conc. of polyanion (μM)	n	Experimental (C_{70}/C_{10})	n	Control (C_{70}/C_{10})
1	139	5	0.36 ± 0.14	7	0.54 ± 0.15
2	33	8	0.50 ± 0.09	7	0.77 ± 0.16
3	139	6	0.49 ± 0.07	9	0.73 ± 0.27

[a] The results are expressed as the ratio of the conductance at −70 mV to the conductance at −10 mV C_{70}/C_{10} for each individual mitochondrion. Control: absence of polyanion

range. The order is reversed in the positive range. The polyanion was introduced after 3.5 min of incubation. The results summarized in Fig. 8 for both the presence (curve *2*) and the absence (curve *1*) of polyanion, are expressed as relative conductance measured at −200 mV (across both electrode and membrane).

A polyanion-induced decrease in conductance was also observed at steady state, as determined in separate experiments summarized in the Table. For convenience, the results are expressed as the ratio of the conductances at −70 and −10 mV. In effect this procedure normalizes the results and provides a parameter that expresses the response of each patch to the potential change. On average there was a small but significant difference between the controls and the results obtained in the presence of polyanion.

A better indication of what occurred in this experiment is obtained by ranking the individual determinations according to relative conductance (Fig. 9). Clearly, in the presence of the polyanion, there is a dramatic increase in the proportion of

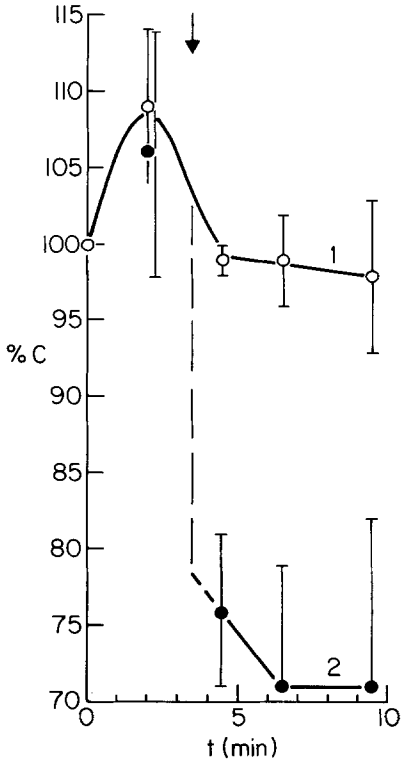

Fig. 8. Time course of the effect of polyanion on the conductance at −200 mV derived from the *IV* curves. No series resistance compensation. The mean electrode resistances were 317 MΩ for the controls and 305 MΩ for the experimentals. The results are plotted as mean ± SD; $n = 4$. Curve *1* (open symbols): controls; Curve *2* (closed symbols): at the arrow 42 μM of the polyanion was added to the medium

mitochondria that respond to increasing negative potentials.

Doring and Colombini (1985*a*,*b*) have shown that treatment of VDAC with succinic anhydride

virtually eliminates the voltage dependence of the channel and modifies its ion selectivity. We have explored the sensitivity of the membranes to treatment with this reagent in the absence of an imposed potential. Oscilloscope tracings of typical results are shown in Fig. 10 in which *a* corresponds to the control and *b* to succinic anhydride-treated mitochondria. After treatment the mitochondrial patches lose their ability to close in response to the negative potentials and instead display an increase in conductance in both negative and positive ranges of potentials.

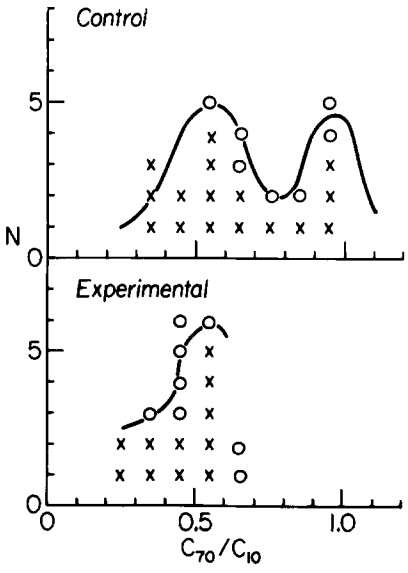

Fig. 9. Ranking of the individual mitochondria as a function of their sensitivity to negative voltages. *N* represents the number of mitochondria (ordinate). The abscissa represents the ratio of conductance at −70 mV (C_{70}) to the conductance at −10 mV (C_{10}). Crosses and circles represents two different polyanion concentrations. In the first set the polyanion concentration (crosses) was 139 μM; in the second it (circles) was 33 μM

Since the pI of the VDAC protein ranges between 7.7 and 7.9 we examined the dependence of the conductance of the native membranes on pH using two pH values spanning the pI (*see* Discussion). After normalizing the conductances in the negative range of potentials by considering the values at −10 mV as unity, those obtained at pH 8.6 were plotted as a function of those at pH 5.9 in Fig. 11. The straight line with a slope of one indicates precise equivalence. The individual voltage is recorded next to each point. The membrane conductances at −10 mV were (mean ± SD) 9.8 ± 5.4 nS (pH 5.9) and 7.9 ± 5.0 nS (pH 8.6). There is no significant difference between any of the values. The results in the positive range of potentials also fail to show an important effect. Since the deviations are high and the number of determinations small, a minor effect cannot be ruled out. The conductance values at 10 and 25 mV (normalized to the conductance at −10 mV for each pH) were 0.75 ± 0.35 and 1.04 ± 0.41 ($n = 4$), respectively, at pH 5.9 and 1.02 ± 0.13 and 1.32 ± 0.28 ($n = 4$) at pH 8.6. The voltages at which there was a steep increase in

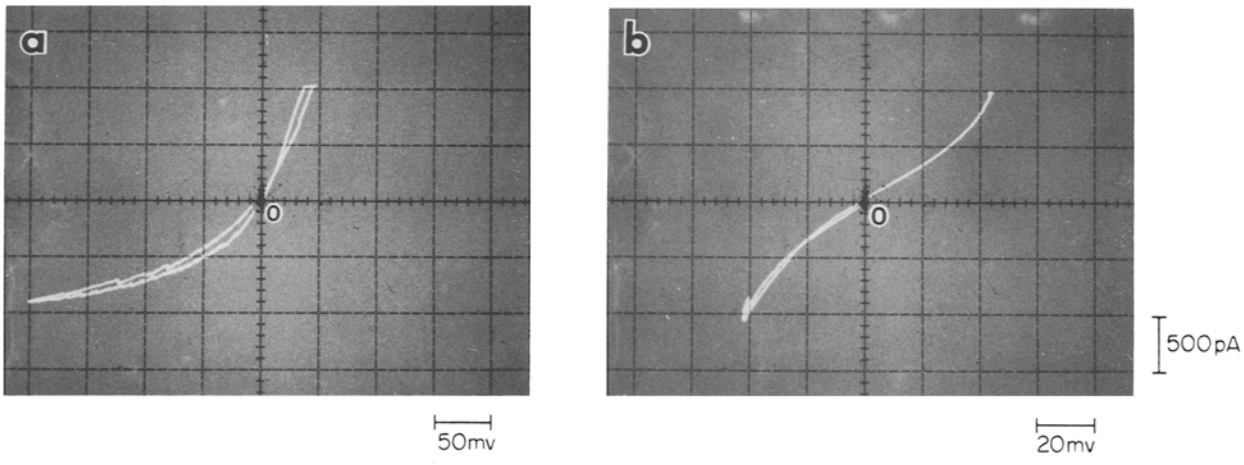

Fig. 10. Effect of succinic anhydride treatment on mitochondria. In both cases shown below, the microelectrode contained our usual medium (*see* Materials and Methods). The final pH of the mixture was 4.5 for both control and experimental. (*a*) Control. *IV* curve of a giant mitochondrion. 40 μl of 8.2% succinic anhydride in DMSO (dimethylsulfoxide) were added to 500 μl of the 50 mM MOPS (3-[N-morpholino]propane sulfonic acid). After 10 min, 100 μl of mitochondrial stock suspension were added. Succinic anhydride should have completely hydrolyzed within this period. Seven independent determinations were carried out. Five of these displayed the pattern the oscilloscope record presented. Two showed no rectification. (*b*) Experimental. *IV* curve of treated mitochondrion. Forty μl of 8.2% succinic anhydride in DMSO were added to a suspension prepared by mixing 500 μl of 0.3 osmolal sucrose, sucrose-MOPS solution, pH 7.5, to 100 μl of stock mitochondrial suspension. Four independent determinations showed essentially the same pattern

conductance resulting in a current greater than 1000 pA were 36 ± 22 mV (n = 10) at pH 5.9 and 22 ± 18 (n = 4) at pH 8.6.

The increase in conductance with increasing positive voltage could conceivably correspond to some nonspecific breakdown of the membrane structure. However, there are two arguments against this view. The voltage-sensitive increase in conductance (right-hand panels of the *IV* curves) is reversible. Furthermore, the conductance increase is enhanced by the polyanion as shown by the results of Fig. 7. The reversibility of the effect of positive voltage is shown most clearly in experiments in which the effect is followed with time as shown in Fig. 12. In this experiment the membrane was clamped briefly at a particular membrane voltage (lower trace), twice. The record of the current (upper trace) shows that the voltage clamping produces current traces that have very nearly the same time course even when the recovery time between the two pulses was not much more than a second.

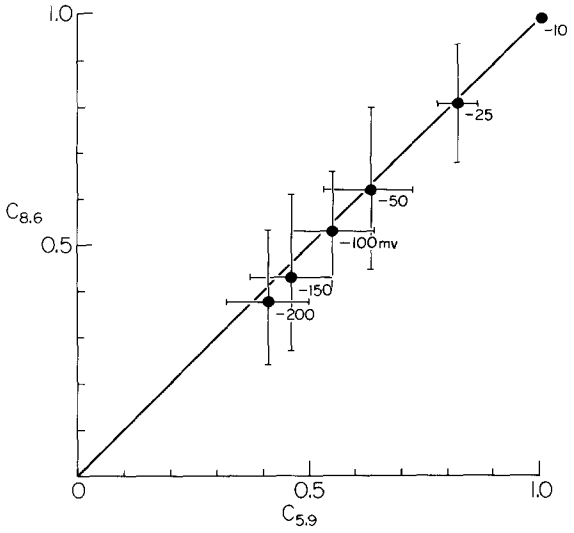

Fig. 11. Comparison of the *IV* curves obtained at pH 8.6 (ordinate) to those obtained at pH 5.9 (abscissa). The results were normalized by considering the conductance at −10 mV as 100%. The potential at which the conductance was calculated is shown for each individual point. For the results in the positive range of potentials, *see* text. The results are means ± SD for pH 5.9, n = 7; for pH 8.6, n = 4

Discussion

In these patch-clamp studies, essentially the same results are obtained regardless of the configuration of the vesicle material in relation to the patching pipette. The *IV* curves remain the same after the rest of the vesicle detaches from the pipette. Furthermore, there is no significant difference between results obtained when a very large membrane piece enters the pipette or when the tip of the pipette contains a small piece normal to the axis of the pipette.

These observations are not surprising since we would expect the major contribution to the resistance to be from the patch. With a large membrane piece, a large portion of the membrane is parallel to the sides of the pipette and is probably fused to the glass. When a whole mitochondrion is patched, we would expect neither the rest of the outer membrane nor the inner membrane to play a significant role. The outer membrane constitutes a much larger

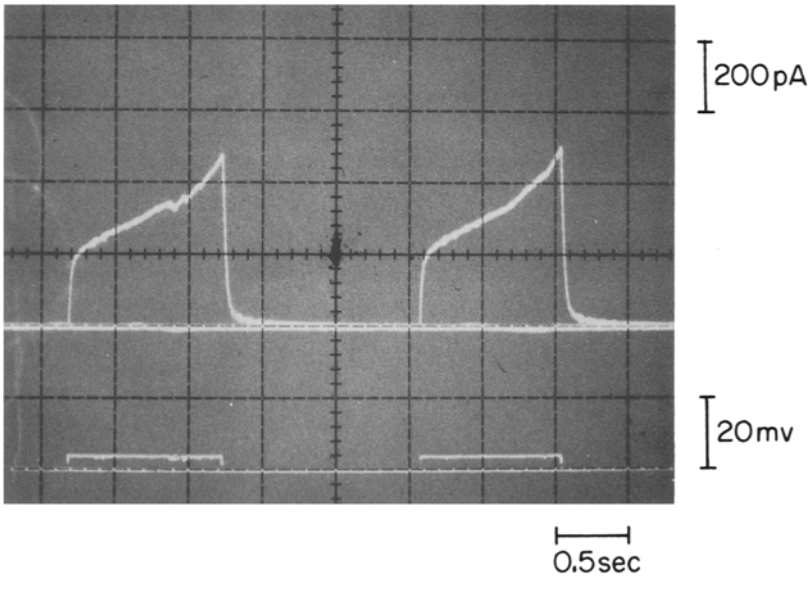

Fig. 12. Oscilloscope trace showing reversibility of the increase in mitochondrial patch conductance produced by positive potentials. The base lines correspond to 0 current and potential. The in-series resistance was compensated for electronically. Top trace: current; bottom trace: voltage

conducting surface area than the patch. In a mitochondrion 5 to 10 μm in diameter, the surface area would be between 80 to 300 μm^2, whereas for a patch on a pipette opening 1.5 μm in diameter, it would be 2 μm^2, i.e., a 40 to 150-fold differential. The space between the inner and outer membrane should reflect the conductance of the external medium, and the much higher resistance expected for the inner membrane which is in parallel to the alternative pathway would not be involved.

Since the results obtained with patch-clamped mitochondria were similar to those obtained with isolated fused outer mitochondrial membrane, we conclude that we are dealing with the outer mitochondrial membrane in both cases.

The use of patch-clamping techniques in the study of the intact mitochondrial outer membrane may have consequences distinct from those of other techniques such as the electrical study in bilayers of channel-forming proteins. The advantage of the patch-clamp techniques is that they allow the study of native membranes and channels in their natural membrane environment. A problem encountered in applying patch techniques to outer mitochondrial membrane is the significant electrical resistance of the micropipette which is in-series with the membrane. This requires a correction as already discussed (*see* Materials and Methods).

The geometry of the membrane in relation to the micropipette may have a bearing on interpretation of these findings. Most frequently, a portion of the membrane parallels the walls of the pipette which contain it and in these locations the electric field is in the plane of the membrane. An electric field parallel to the plane of the membrane should allow the migration of charged integral proteins. Such a migration has been demonstrated in inner mitochondrial membrane preparations (Sowers & Hackenbrock, 1981). Conceivably, a negatively charged channel protein could migrate away from the patch when the applied voltage is negative (i.e., negative inside the pipette) and into the patch when positive. Therefore, the migration of a negatively charged pore protein could produce the conductance changes described, at least qualitatively. However, a major role for migration of the VDAC in the outer mitochondrial membrane patches is not likely. In our data, the effect of a positive potential is sometimes complex, including a decrease in conductance over part of the range and an increase at higher voltages. This is not the behavior we would expect from the electrophoretic migration of a single population of channels.

This proposition can be put to a more rigorous test. Since the VDAC polypeptide has a pI of approximately 7.7 for *Neurospora* (Freitag et al, 1982) and 7.9 for liver mitochondria (Linden et al., 1982), we compared the results at pH 8.6 and 5.9 and found them indistinguishable (Fig. 11). Since VDAC should be oppositely charged at the two extremes of pH, these results argue against a role electrophoretic migration of VDAC in the effects observed. The charge at one pH should reverse in sign at the other. Bowen et al. (1985) have shown previously that pH in this range has virtually no effect on the functional characteristic of VDAC in bilayers.

In the patch studies of the outer mitochondrial membrane, the density of the channels is also significantly higher than that of the previous bilayer studies. In bilayers the maximum number of channels corresponds to several hundred per mm^2, in membranes a similar number per μm^2. Any cooperative interactions between the channels, suggested, for example, by their tendency to crystallize in the plane of the membrane (Mannella, 1982), would be more likely to be detected in the native membranes.

The decrease in conductance with changes in voltage most likely corresponds to closing of channels, like that observed with VDAC in bilayers. Furthermore, this apparent closure is increased by the polyanion inhibitor as found for the bilayer system. We have found that the proportion of the mitochondrial patches that does not respond to voltage (i.e., does not exhibit rectification) is induced to respond by the presence of the polyanion. This latter observation seems to be in harmony with the proposal of Yeung et al. (1986) that the polyanion mimics some natural regulatory factor. Our results suggest that the polyanion confers voltage sensitivity on some patches by replacing a lost endogenous factor.

The closing of the channels in the negative range of voltages was blocked by treatment of the membranes with succinic anhydride, as previously observed with VDAC in bilayers by Doring and Colombini (1985*a*,*b*). However, in our experiments the effect of the treatment was more complex since it induced an increase in conductance in the negative range of potentials. At the same time, the voltage-dependent increase in conductance remained approximately the same in the positive range of potentials. It would be interesting to consider that in the treated mitochondria, the effects of positive and negative potentials may reflect the same underlying phenomenon. Succinic anhydride is thought to replace amino groups with carboxyl groups. Any proposed mechanism would have to take into account possible contribution of carboxyl groups in eliciting this effect in the negative range of potentials.

The number of channels per patch can be calculated on the assumption that the conductance of one channel corresponds to that of one open VDAC in-

corporated into a bilayer. A single VDAC has been calculated to have a conductance of approximately 4.5 to 5 nS when the conducting medium is 1 M KCl. Since the dependence of VDAC conductance on KCl concentration is linear, each VDAC in our medium (10 mM KCl) should have a conductance 100 times lower. The conductance calculated for our patches ranges from 5 to 50 nS at −10 mV for either mitochondria or outer membrane vesicles. Therefore there should be from 100 to 1000 channels per patch, generally consistent with the surface density of VDAC polypeptide (Freitag et al., 1982). These values, however, are unlikely to accurately reveal an upper limit for channel density. In our experiments we have found it hard to detect and record *IV* curves from highly conducting patches. Such patches have a resistance which is negligibly higher than that of the electrode alone. Furthermore, at the ionic strengths used in this study the electrode resistance fluctuates slightly even in the absence of a membrane patch, hindering any meaningful recordings from patches of extremely low resistance.

The increase in conductance in the positive range of potentials found in the untreated membranes could correspond to: (a) breakdown of membrane components, (b) the presence of a class of channels not previously described, (c) some structural rearrangement of VDAC, and (d) the electrophoretic displacement of channels into the patch. As described above, the last alternative is unlikely because the observed effects are pH independent. Similarly, the first possibility, that of membrane breakdown, seems to be unlikely in view of the reversibility of the effect and asymmetry in relation to potential. While none of these possibilities can be formally eliminated at this time, we favor a role of VDAC since the effect is reversible and sensitive to the polyanion which is a known affector of VDAC.

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