Measurement of VDAC permeability in intact mitochondria and reconstituted systems

Marco Colombini

Department of Biology, University of Maryland

College Park, MD 20742

Voice: 301-405-6925 Fax: 301-314-9358

e-mail: Colombini@umd.edu

Running title: Measurement of VDAC permeability

Abstract

VDAC channels are located in the mitochondrial outer membrane. They control metabolite flux between mitochondria and the cytosol. Moreover, changes in the conducting state of VDAC have been implicated in the initiation of mitochondria-mediated apoptosis. Thus methods to monitor the conducting state of VDAC should be useful in investigating these important functions. The permeability of the outer membrane is dominated by VDAC and thus measuring the permeability of this membrane is a measure of the state of VDAC channels in the intact mitochondrion. Three methods are described to measure the permeability of the outer membrane to metabolites. These all take advantage of mitochondrial enzymatic activity and the rate limitation of the outer membrane. In order to characterize the detailed properties of VDAC, the protein needs to be purified and reconstituted into phospholipid membranes for electrophysiological analysis. A discussion of this method is presented, pointing out the pitfalls to avoid in making these measurements.

I. Introduction

Since the early work of Werkheiser and Bartley (1957), mitochondria have been known to consist of 2 membranes: the outer and the inner membrane. In the early days, mitochondrial research focused primarily on energy transduction and the characterization of isolated mitochondria focused on criteria related to coupled energy transduction such as the respiratory control ratio. In those days the function of the outer membrane and the molecular basis for its permeability were not understood or appreciated. However, times have changes and the importance of the outer membrane is no longer in question. It is literally a question of life and death for the cell. Among the roles of the outer membrane is the regulation of the permeability of the outer membrane to metabolites. This role is the function of VDAC (Colombini, 1979) , the channel by which metabolites cross the outer membrane.

VDAC is a 32kDa protein that forms monomeric channels in the mitochondrial outer membrane of all eukaryotes tested (Mannella et al., 1992; Colombini, 1994). Where multiple isoforms exist, at least one isoform, called VDAC1, has properties that are virtually identical irrespective of the source (Colombini, 1989). This includes two voltage-gating processes, preference for anions, and single-channel conductance. The variation in the properties of other isoforms is believed to be related to specialized functions in that organism or cell type.

Isolation of mitochondria typically involves the homogenization of the cell. This traumatic experience results in some damage to organelles. Mitochondria are no exception. If mitochondria in that cell exist as a mitochondrial reticulum (one large reticulated mitochondrion) the structure will be fragmented. When many mitochondria

are present, less damage is likely. In any case, the outer membrane is most vulnerable not only because it is the mitochondrial surface but also because its surface area is much smaller than that of the inner membrane. Swelling of the inner membrane can damage the outer membrane without breaching the integrity of the inner membrane. Thus assessing the intactness of the outer membrane is essential.

Beyond assessment of mitochondrial integrity, characterization of mitochondria should include an assessment of the status of the VDAC channels in the intact mitochondrion. VDAC channels are the pathway by which metabolites cross the outer membrane and thus the permeability of the outer membrane to metabolites reflects the state of VDAC. A variety of conditions can influence the probability of VDAC closure (Colombini, 2004). Thus characterizing the permeability of the outer membrane to metabolites provides information on the state and history of the mitochondrion.

Permeability measurements must go beyond the determination of absolute permeability to the determination of rates of permeation. Mitochondria are dynamic structures capable of high rates of metabolism. Thus the rate at which metabolites can cross the outer membrane will limit mitochondria metabolic rates which will also greatly influence metabolic rates in the rest of the cell because of the highly intertwined nature of the two metabolic systems. The extensive impact of altering the permeability of the outer membrane has been explored in a recent publication (Lemasters and Holmuhamedov, 2006).

II. Determining the Permeability of the Mitochondrial Outer Membrane to Metabolites

A. Rationale:

Since VDAC is responsible for metabolite flux across the outer membrane, determining the permeability of the outer membrane to metabolites also determines the state and function of VDAC in the intact organelle. However, the small volume of the intermembrane space and the high permeability of the outer membrane to metabolites make metabolite flux measurements across the outer membrane very difficult. A solution is to take advantage of enzymatic activity to consume the metabolite once it has crossed the outer membrane. If the rate of the enzymatic reaction were limited by the rate of metabolite translocation across the outer membrane then the permeability of that membrane to the metabolite could be calculated. Changes in this permeability following any treatment would provide quantitative information of the influence of the treatment on the open-channel probability of VDAC.

B. Assay of Outer Membrane Intactness

Outer membrane intactness must be assessed first to estimate the fraction of mitochondria with damaged outer membrane. This is measured by the method of Douce et al., (1987). Intact outer membranes are impermeable to cytochrome c. Following outer membrane damage, exogenously added cytochrome c can access enzymes in the inner membrane. The measurement of KCN-sensitive cytochrome c oxidation by complex IV (cytochrome oxidase) is convenient. This can be done either by following the drop in absorbance of reduced cytochrome c at 550 nm or by measuring the rate of oxygen consumption using an oxygen electrode (e.g. Lee and Colombini, 1997).

C. Methods:

1) Determination of the permeability of the outer membrane to ADP from respiration measurements (Lee et al., 1994; Lee and Colombini, 1997)

a) Theory:

The permeability of the outer membrane to ADP can be extracted from measurements of ADP-dependent respiration. ADP-dependent respiration by isolated mitochondria requires 4 processes in series:

1. ADP diffusion through VDAC channels in the outer membrane
2. ADP/ATP exchange through the inner membrane
3. phosphorylation by the ATP synthase
4. proton pumping through the respiratory chain coupled to oxygen consumption

At steady state process (1) and (2) must proceed at the same rate. The experiments must be run under conditions where steps (3) and (4) are not rate limiting. If this condition does not hold true then the measured values of the permeability are only apparent but still useful for comparison purposes with appropriate controls.

Thus, at steady state, the rate of depletion of medium [ADP]:

$$\frac{d[ADP]}{dt} = -\frac{P([ADP]_o - [ADP]_i)}{Vol} = -\frac{V_{\max}[ADP]_i}{K_M + [ADP]_i} \qquad (1)$$

where P is the total permeability of the outer membrane, Vol is the volume of the medium, and K_M and V_{max} are the kinetic parameters of the adenine nucleotide translocator and “o” and “i” refer to the outside compartment (the medium) and the intermembrane space resp. Note that the units of V_{max} are not the standard units but concentration per unit time. Solving for $[ADP]_i$:

$$[ADP]_i = \frac{-\left(\frac{V_{\max} Vol}{P} + K_M - [ADP]_o\right)}{2} + \sqrt{\left(\frac{\frac{V_{\max} Vol}{P} + K_M - [ADP]_o}{2}\right) + K_M [ADP]_o} \quad (2)$$

This equation needs to be fit to the experimental results to extract a value for the permeability, P. The experimental results begin as the medium oxygen concentration as a function of time following the addition of a fixed amount of ADP (Fig. 1, left panel). By subtracting state IV from the respiration curve one achieves a relationship for the ADP-dependent oxygen consumption. We must assume that the ADP-independent oxygen consumption is not altered by the addition of ADP. This may introduce a small error because of the change in membrane potential upon ADP addition. However the error should be small especially since state IV respiration is much lower than state III. Using the P/O ratio (2 for succinate) one can convert the curve from oxygen concentration as a function of time to [ADP] as a function of time (Fig. 1, right panel). Now the theoretical relation can be used to fit the theory to the data.

A theoretical curve can be generated for arbitrary values of P, V_{max}, and K_M, by calculating new values of $[ADP]_o$ for each time increment dt (matching the time increments in the digitized data). Thus:

$$[ADP]_o^{t+dt} = [ADP]_o^t + \frac{d[ADP]_o}{dt} dt = [ADP]_o^t - \frac{V_{\max} [ADP]_i^t}{K_M + [ADP]_i^t} dt \quad (3)$$

For details on two methods of fitting the data see Lee and Colombini (1997). A unique fit can sometimes be achieved without the need for additional information. However, the K_M value can be obtained on the same set of mitochondria in a separate experiment on hypotonically-shocked mitochondria. The same fitting is performed except that the P value is given a very large number, say 1000. In our experience this worked well for

mitochondria from potatoes but not from mitochondria from rat liver. Damage of the outer membrane of rat liver mitochondria changes the respiration properties. Even rather mild osmotic shock (100 mOsmolar for 10 minutes on ice) caused changes that cannot be attributed merely to outer membrane damage. An alternative way to reduce the number of variables is to set the K_M to the published value of 4 µM (Pfaff et al., 1968) for the adenine nucleotide translocator. This will yield relative values of permeability, before and after some experimental treatment.

b) Equipment

Clark oxygen electrode set-up: If this unit has an analogue output then the data should be digitized into a good-quality A/D converter (at least 10 bits). If it has a digital output than this can be fed directly into a computer and processed using the manufacturer's software. Example: Yellow Springs Instruments; Mitocell S200 from Strathkelvin Inst.

Analog to Digital converted (optional, see above): A good quality unit such as one used for electrophysiological recordings will work well. Example: Axon Instruments Digidata 1322A

Chart recorder (optional): It is convenient to also record the oputput from the oxygen electrode into a chart recorder so that one can more easily keep track of the experimental results. Example: Kipp and Zonen BD12E

c) Reagents

10 mM ADP

100 mM Na_2succinate

Respiration buffer: 0.3 M mannitol, 10 mM NaH_2PO_4, 5 mM $MgCl_2$ and 10 mM KCl (pH 7.2)

d) Protocol

Add mitochondria to 3 ml (volume depends on instrument used) of respiration buffer in the oxygen electrode chamber under temperature control. Room temperature works well. The total mitochondrial protein used is generally about 1 mg. Begin recording oxygen level. Add 150 µl succinate (5mM final) and record briefly to see measurable rate. Add 27 µl ADP (final 90 µM) and record state 3/state 4 respiration transition. Record until constant state 4 respiration is achieved. Then repeat ADP addition 3 more times. The result of the first addition may be unreliable as the mitochondria adjust to the experimental protocol but the subsequent 3 trials (before the oxygen level runs out) should be virtually identical. This shows good-quality, stable mitochondria. The last 3 records are analyzed to determine the permeability.

The data collected for each state3/state4 transition should be converted into a data file. If the Axon Instruments Digidata A/D converter is used along with the Clampfit software, one can easily "cut-out" the appropriate part of the record and paste it into a spreadsheet like Excel. The data can now be saved as a "csv" file and imported into a program to fit the data to the theory. A QBasic program we developed for this purpose is available for free download from "www.life.umd.edu/biology/faculty/colombini/"

2. Measurement of the permeability of the outer membrane to NADH from measurements of NADH oxidation

a) Rationale

Classical state III/state IV respiration curves can be obtained from mammalian mitochondria and from those of higher plants but these cannot be obtained by using mitochondria from the yeast, *S. cerevisiae*. Addition of ADP to mitochondria isolated from this yeast results in an increase in respiration that does not return to baseline for the length of the experiment. Addition of ATP yields similar results. Thus the method described in part 1 cannot be used to determine the permeability of the outer membrane to metabolites. Instead one can take advantage of another peculiarity of yeast mitochondria to measure the permeability of the outer membrane to the metabolite, NADH. Unlike mammalian mitochondria, yeast mitochondria have an NADH dehydrogenase on the outer surface of their inner membrane and thus can use cytosolic NADH and deliver the electrons to cytochrome oxidase and thus to oxygen.

b) Procedure (Lee et al., 1998)

The NADH concentration is measured as function of time by recording the absorbance at 340 nm. This is done for both intact mitochondria and hypotonically-shocked mitochondria (Fig. 2, left panel) . Simultaneous recordings at 400 nm can be used to detect any volume changes in the mitochondria that could affect the measured values at 340 nm. The rate of NADH oxidation is obtained from these oxidation curves by calculating the slope of the tangent at each point. This can be done by determining the least squares fit on all groups of adjacent 13 points by processing the data in a spreadsheet. The slope, m, is given by:

$$m = \frac{\sum xy - n\bar{x}\bar{y}}{\sum x^2 - n\bar{x}} \qquad (4)$$

where x and y are the [NADH] and time for each data point, $\bar{x}$ and $\bar{y}$ are the mean values, and n is the number of points used to fit the line. This equation can be written in individual cells in the spreadsheet to obtain the slope for each point (except the first and last 7 points) and thus the corresponding rates of NADH oxidation. Note that these rates are in concentration per unit time. The number of points to be used to determine the slopes can be determined by trial and error although 13 worked well for us. The greater the number of point the less the noise but if too many points are chosen it is equivalent to filtering the data and the values of the rates are reduced. We chose a number of points that did not reduce the calculated rates.

The rate of NADH oxidation depends on the medium [NADH] and thus the calculated rates are plotted against the [NADH] in the medium (Fig. 2, right panel). From these plots one can obtain the permeability of the outer membrane. The relationship is as follows:

$$\frac{d[NADH]_o}{dt} = \frac{P([NADH]_o - [NADH]_i)}{Vol} \qquad (5)$$

where "o" and "i" refer to the outside compartment (the medium) and the intermembrane space resp. Thus the only value needed in order to calculate the permeability, P, is the intermembrane space concentration of NADH. This can be obtained by using the results from the shocked mitochondria (damaged outer membrane) if one assumes that the rate of NADH oxidation depends on the [NADH] in the vicinity of the enzyme.

A ninth order polynomial fit of the data relating the measured NADH oxidation rate of shocked mitochondria and the measured medium [NADH] yields an equation that converts oxidation rates to [NADH] close to the enzyme. This equation can then be used

to convert the rates of oxidation measured with intact mitochondria to the intermembrane concentration of NADH. This is illustrated in Fig. 2, right panel. For each point on the curve for intact mitochondria has its associated $[NADH]_o$ but the $[NADH]_o$ that resulted in the same respiration rate in shocked mitochondria is the $[NADH]_i$ for the intact mitochondria. Ideally one could just determine this graphically as shown in the figure but, because of noise in the data it must be obtained from the 9^{th} order polynomial equation. This is also much faster that determining each $[NADH]_i$ graphically. With these values, one has all the parameters needed to calculate a value of P for each data point using Eq. (5).

c) Procedures for isolating mitochondria and obtaining shocked mitochondria from yeast

Mitochondria were isolated from yeast by methods designed to maximize the intactness of the outer membrane (Lee et al., 1998). Mitochondria with damaged outer membrane were generated as previously described (Lee et al., 1998) in order to minimize degradation in function. An aliquot of the mitochondrial suspension was diluted with 2 volumes of ice-cold distilled water and allowed to incubate on ice for 10 min. Then 5 times the original volume of cold medium was added. Finally 2 times the original volume of cold 2-fold concentrated medium was added. The shocked mitochondria were finally back in the original medium but diluted 10 fold.

Reagents:

3 mM NADH

Oxidation buffer: 0.65 M sucrose, 10 mm HEPES, 10mM phosphate, 5mM KCl, 5mM $MgCl_2$, pH 7.2 with KOH.

Protocol:

Add mitochondria to 1 ml (volume depends on cuvette used) of oxidation buffer in a cuvette (25 µg mitochondrial protein). Allow to equilibrate at room temperature for about 20 min (to stabilize volume…follow at 600 nm until there is no change in absorbance). Add 40 µL of NADH (30 µM final) and record absorbance at 340 nm. Continue recording until there is no further decline in absorbance. Repeat the experiment with the shocked mitochondria.

3. Measurement of the permeability of the outer membrane to ATP in yeast mitochondria using adenylate kinase activity

a) Principle of the method: (Komarov et al., 2005)

Adenylate kinase catalyzes the reaction:

2 ADP $\longrightarrow$ ATP + AMP

This enzyme is located in the mitochondrial intermembrane space and thus the rate of reaction depends on the permeability of adjacent membranes to substrates and products. If the adenine nucleotide translocator were inhibited by using atractyloside (20 µg/mL), then VDAC channels in the outer membrane would be the only pathway for metabolite access to this enzyme.

The situation is complicated by the necessity to add enzymes to couple the reaction to a detectable product, NADPH. These enzymes, hexokinase and glucose-6-phosphate dehydrogenase, are located in the medium, separated from adenylate kinase by the outer membrane. Thus to generate one molecule of NADPH, two molecules of ADP must move through VDAC from the medium and be converted to AMP and ATP in the

intermembrane space. The ATP must return to the medium to be used by hexokinase to phosphorylate glucose. The resulting glucose-6-phosphase is then used by the dehydrogenase to convert NADP to NADPH. The latter is detected by an increase in absorbance at 340 nm. To inhibit oxidation of NADPH, the medium was supplemented with 0.2 mM KCN.

The system must be run in such a way that the coupled enzyme reactions do not limit the rate of adenylate kinase activity. Under these conditions, the reaction can be limited by the rate of entry ADP into and exit of ATP from the intermembrane space.

b. Determination of the permeability to ATP

At steady state, the rate of NADPH production must be equal to the net efflux of ATP from the mitochondrion and also equal to one half the net influx of ADP (note the stoichiometry of adenylate kinase). The $[ADP]_o$ at any time during the reaction is known because it must be equal to the initial concentration minus the [NADPH] that is measured continuously (absorbance at 340 nm). Therefore:

$$\frac{d[NADPH]}{dt} = -\frac{d[ADP]}{dt} = \frac{0.5P_D\left([ADP]_o - [ADP]_i\right)}{Vol} = \frac{P_T\left([ATP]_i - [ATP]_o\right)}{Vol} \qquad (6)$$

Vol is the volume of the medium. To determine the permeability of the outer membrane to ADP, P_D, we need to know $[ADP]_i$, the [ADP] in the intermembrane space. The medium [ATP] is kept essentially at zero through of the action of hexokinase. Thus to obtain the permeability of the outer membrane to ATP, P_T, we need to know the [ATP] in the intermembrane space, $[ATP]_i$. The approach used in section 2 does not work here because the rate of the reaction depends on both substrate and product concentrations. Damaging the outer membrane results in obtaining a rate in the presence of medium

[ADP] but also virtually zero [ATP] (due to the action of hexokinase). This rate cannot be used to measure the $[ADP]_i$ because the [ATP] is changing.

However, the unknown concentrations can be estimated from the kinetic properties of adenylate kinase. The net forward rate of this enzymatic activity will be determined by the $[ADP]_i$ and reduced by $[ATP]_i$. These effects must be consistent, and indeed are constrained by the known kinetic properties of the enzyme. In order to reduce the unknowns, one assumes that the permeability of the outer membrane is the same for ADP, ATP and AMP. Since the goal is to assess the permeability of the outer membrane to metabolites, this assumption is reasonable. With this assumption and Eq. (6) it follows that:

$$[ADP]_o - [ADP]_i = 2[ATP]_i = 2[AMP]_i \qquad (7)$$

This is not quite right for the $[AMP]_i$ except at initial times. However the use of initial rates largely eliminates this problem.

Kinetic studies of yeast adenylate kinase (Su and Russell, 1968) have shown that the reaction is limited by the rate of conversion of the enzyme from E-ADP_2 to E-AMP-ATP. Thus, the binding steps in the adenylate kinase reaction can be assumed to be at equilibrium. Thus:

$$v_o = k_f[E(ADP)_2] - k_r[E(ATP)(AMP)] \qquad (8)$$

for the reaction in intact mitochondria. v_o is the initial rate and k_f and k_r are the forward and reverse rate constants, resp. For the shocked mitochondria the second term is zero. Substituting for the unknowns:

$$v_o = \frac{V_{\max}^f K_D^2 [ADP]^2 - V_{\max}^r K_M K_T [ATP]^2}{1 + K_D[ADP] + K_D^2[ADP]^2 + K_T[ATP] + K_M[ATP] + K_M K_T[ATP]^2} \quad (9)$$

where K_D, K_T, and K_M are the equilibrium binding constants for ADP, ATP, and AMP, resp. For yeast mitochondria these are: 3700, 19,000, and 17,000, resp. V_{max}^f and V_{max}^r are the maximal velocities in the forward and reverse directions, resp. The equilibrium constants are obtained from the reciprocal of the Michaelis constants (Su and Russell, 1968) and the ratio of the V_{max} values is known to be 0.6 at neutral pH (Chiu et al., 1967). Additional expressions needed to solve for the needed concentrations numerically are:

$$Ratio = \frac{V_{\max}^f}{V_{\max}^r} \quad (10)$$

$$V_{\max}^f = \frac{v_o^{shock}(1 + K_D[ADP]_o + K_D^2[ADP]_o^2}{K_D^2[ADP]_o^2} \quad (11)$$

$$v_o = \frac{d[NADPH]}{dt} Vol \quad (12)$$

where v_o^{shock} is the initial velocity measured for shocked mitochondria. Note that here the [ADP] is different from that in Eq. (8) because the mitochondria are shocked and thus the enzyme is in the medium.

The equations are easily solved with the aid of a spreadsheet program or math program. The unknown $[ADP]_i$ can be replaced, using Eq. (6), by the known medium concentration and the $[ATP]_i$, thus eliminating one of the unknowns. The initial rate for shocked mitochondria is used to calculate V_{max}^f using Eq. (11). Then an $[ATP]_i$ is selected for Eq. (8) that yields an initial velocity for adenylate kinase in intact

mitochondria that matches the measured value. From this value the other concentrations can be calculated along with the permeability to nucleotides.

c) Reagents:

12.5 mM ADP

hexokinase, and glucose-6-phosphate dehydrogenase dissolved in water at 1 unit per µL (can be stored frozen and survives freeze thawing)

Reaction medium: 0.6M sucrose, 50mM TrisCl, 5mM $MgSO_4$, 10mM glucose, 0.2mM NADP, 20mg/ml atractylosides, 0.2mM KCN, pH7.5

2 Fold reaction medium: 1.2M sucrose, 100mM TrisCl, 10mM $MgSO_4$, 20mM glucose, 0.4mM NADP, 40mg/ml atractylosides, 0.4mM KCN, pH7.5

d) Protocol for yeast mitochondria

Mitochondria are isolated from yeast by methods designed to maximize the intactness of the outer membrane (Lee et al., 1998). These mitochondria (10–14mg protein/mL) are diluted 40-fold with reaction medium (room temperature) just before use. Shocked mitochondria are generated by mixing the initial mitochondrial suspension with 9 volumes of ice-cold distilled water and incubating on ice for 5min. Then 10 volumes of 2-fold reaction medium (at room temp) are added followed by 20 volumes of reaction medium (room temp). This results in the same 40-fold dilution and restores the original solute concentrations.

To 1 ml of mitochondrial suspension, add 20 µL of ADP (250µM final concentration), and 10 µL of the hexokinase/glucose-6-phosphate dehydrogenase mixture

(10 units each). The increase in [NADPH] is followed by recording the absorbance at 340 nm. There may be an initial fast increase in absorbance due to the presence of a small amount of ATP in the ADP solution. (see a fast initial increase in Fig. 3) A 1% contamination produces an obvious effect. Thus the initial rate is assessed after the initial fast increase. By overlapping the curves obtained with intact and shocked mitochondria one can immediately get a sense of the level of permeability of the outer membrane to ATP. Figure 3 shows an example of data collected with intact and shocked mitochondria from the yeast, *S. cerevisiae*, lacking VDAC (nul) and yeast expressing VDAC (VDAC). The theory extracts the numerical values.

D. Controls

The methods described above are designed to assess the permeability of the mitochondrial outer membrane to metabolites and, in doing so, to probe the functional state of VDAC. The changes that occur upon outer membrane damage provide some confidence that the measurements provide the desired information. However, further evidence exists. The knock-out of VDAC1 in yeast drops the permeability measured by a factor of 20 (Lee et al., 1998). The remaining permeability could be attributed to damaged mitochondria. A recently discovered blocker of VDAC drops the permeability of the outer membrane (measured by method 1) of rat liver mitochondria by a factor of 6 to 7 (Tan et al., under review). These findings provide further evidence that VDAC is the pathway responsible for the permeability of the outer membrane. They also help to validate the methods.

III. VDAC Activity after Reconstitution into Phospholipid Membranes

A. Rationale

The detailed properties of VDAC can only be determined after purification and reconstitution into phospholipid membranes. Electrophysiological recordings of single channels allow one to determine single-channel conductance and selectivity. Recordings on small populations of channels yield the best estimates of voltage-dependent parameters. Effective pore size is best estimated from experiments with liposomes. Studies on reconstituted VDAC channels also allow one to study the influence of test molecules or macromolecules on the properties of VDAC.

B. Purification of VDAC

VDAC channels have been successfully purified from a wide variety of mitochondria by the method of Freitag et al. (1983). This rapid, one-step procedure, yields a sufficient level of purity for functional, reconstitution experiments. Higher levels of purity can be obtained by additional purification steps (Gincel et al., 2000).

At this writing I'm unaware of a satisfactory way of obtaining VDAC with normal function by refolding denatured protein obtained from inclusion bodies. One can obtain channel-forming activity but the properties are different from those observed for VDAC purified from mitochondria. The properties change markedly depending on the method used to refold the protein.

1. Reagents:

Shock medium: 1 mM KCl, 1 mM HEPES pH 7.5

Solubilization medium: 2.5% Triton X100, 50 mM KCl, 10 mM Tris, 1 mM EDTA, 15% DMSO, pH 7.0

Siliconizing solution such as Sigmacote

1:1 dry mixture of hydroxyapatite and celite

2. Protocol:

Mitochondria are hypotonically shocked to remove soluble proteins from the intermembrane and matrix space. The mitochondrial pellet is suspended in about 50 fold excess of cold shock medium and the membranes pelleted at 24,000g for 20 min. These membranes can be stored for extended periods at -80°C if suspended in the shock medium supplemented with 15% DMSO. The pellet (about 1 mg of protein) is resuspended in 0.7 mL solubilization medium and incubated on ice for 30 min with occasional vortexing. The suspension is centrifuged (generally 14,000 g for 30 min in a microfuge) at 4°C to remove non-solubilized material. The supernatant is applied to 1ml of dry hydroxyapatite/celite in a Pasteure pipette (end plugged with a small amount of glass wool and siliconized) at 4°C. The effluent is collected and another 0.7 mL of solubilization medium is applied to the column. The second effluent is mixed with the first and the solution is aliquotted and stored at -80°C. Some VDAC proteins have cysteine residues and so 1mM DTT could be added to the above solutions.

C. Reconstitution of VDAC into Planar Membranes

1. General considerations:

Electrophysiological recordings of VDAC channels have traditionally been made on channels reconstituted into planar phospholipid membranes (Schein et al., 1976;

Colombini, 1979). Recordings with patch-clamp techniques of VDAC in liposomes have been made (Wunder et al., 1991) but VDAC channels have a tendency to rapidly disappear from the patch. Patch-clamp recordings of mitochondrial outer membranes reveal channels with properties that differ from those of purified VDAC (Tedeschi et al., 1989; Jonas et al., 2005). The reason for this is unclear but may have to do with the presence of associated proteins. In one case, VDAC was isolated along with what appeared to be a tightly-bound protein. Protease treatment converted the properties of this reconstituted channel to more classical VDAC behavior (Elkeles et al., 1997).

Generally VDAC needs to be solubilized in detergent (mild non-ionic detergent) to be reconstituted into planar membranes. However, reconstitution without detergent results in properties that are indistinguishable from those seen when detergents are used (Schein et al., 1976).

2. Equipment:

Teflon chamber: Although some chambers are available commercially (e.g. Warner Instruments) the ones used to make membranes from monolayers are made by special order. Contact the author for details.

Electrodes: Mini calomel electrodes from Fisher Scientific work well. They need to be matched so that their electrode asymmetry is 1 mV or less. They can also be made from silver wire by coating them with AgCl and then generating a KCl bridge with 3M KCl.

Dissecting microscope mounted on stand with side arm to observe the formation of the membrane

Stimulator, recording amplifier, filters, etc.: Various systems from Axon Instruments, Warner Instruments, World Precision Instruments, etc.

Faraday cage: wire mesh cage either home made or purchased commercially

Air table: optional for VDAC recordings because of the large single-channel conductance

3. Reagents:

Lipids must be of high quality. All lipids should be purchased from Avanti Polar Lipids. For VDAC studies, a lipid mixture that resembles the lipid composition of the mitochondrial outer membrane is the polar extract of soybean phospholipids (AKA asolectin). Cholesterol is added separately. Often diphytanoyl phosphatidylcholine (DPhPC) is also added to improve membrane stability. A good mixture for easy membrane formation is 1% phospholipid (DPhPC: asolectin; 1:1) 0.1% cholesterol in hexane.

Coating solution: 20% petrolatum in petroleum ether

4. Protocol:

Planar membranes can be made by a variety of published methods. Variations of the monolayer method of Montal and Mueller (1972) works very reliably (Colombini, 1987). A schematic of the set-up is shown in Fig. 4. Two identical Teflon hemichambers are clamped together and squeeze between them a thin partition made of Teflon or Saran. In this partition there is a hole, generally 0.1 mm in diameter. The partition is freshly coated with a thin layer of petrolatum (high molecular weight hydrocarbon) from a solution of petrolatum in petroleum ether. The petroleum ether is allowed to evaporate for 5 min. Syringes are used to inject the aqueous solution into each hemichamber just to

completely cover the floor of the chamber. To form the monolayers, generally 25 µL of the lipid solution is layered on top of the aqueous solution in each hemichamber and the hexane is allowed to evaporate resulting in a small visible nodule of excess lipid floating on the surface of the water. The level of the trans compartment is raised, by injecting solution into the subphase, until the monolayer is just above the hole. Then the level of the cis compartment is raised to generate the bilayer, the planar membrane.

VDAC purified as described in III.B.2. is added directly to the aqueous phase bathing the planar phospholipid membrane. Typically 1 to 4 µL of the VDAC solution is added to 5 mL of aqueous solution while the solution is being stirred with a Teflon-coated magnetic bar. VDAC channels insert spontaneously. Warming the VDAC solution to room temperature before the addition seems to help the insertion process. Typically the voltage is clamped at 10 mV during the insertion period. Depending on the source of VDAC and its concentration, insertion of channels can occur almost immediately or may require an hour or more.

The insertion efficacy is influenced by many variables. Some sources of VDAC insert more readily than others. VDAC tends to insert more easily in the presence of high salt concentrations such as 1 M salt. The amount of detergent added also influences the probability of insertion. The detergent, Triton X100, is particularly effective at facilitating VDAC insertion. The presence of VDAC in the membrane catalyzes the insertion of VDAC. VDAC can autocatalize and control the orientation of the insertion process (Zizi et al., 1995; Xu and Colombini, 1996).

D. Electrophysiological Recordings

1. General Considerations:

Basic electrophysiological characterization of VDAC involves three measurements: single-channel conductance, reversal potential as a measure of selectivity, and voltage dependence parameters. All these measurements can be made under an almost limitless variety of conditions. If one wants to compare with literature (e.g. Colombini, 1989) values then the most common conditions are: single channel conductance and voltage dependence measurements in 1.0 M KCl; reversal potential measurement in the presence of 1.0 M KCl vs. 0.10 M KCl. Generally the solutions also contain some buffer and divalent salts. VDAC selectivity values (permeability ratios for anions over cations) are much higher if measured in lower salt concentrations and shallower salt gradients. Selectivity values are also influenced by the choice of membrane lipid, especially the presence of charge. The voltage-dependence parameters: the steepness of the voltage dependence, n, and the voltage at which half the channels are closed, V_0, are strongly influenced by the presence of a salt gradient (Zizi et al., 1998).

The voltage gating of VDAC involves two distinct gating processes, one at positive potentials and the other at negative potentials. The channels are open at low potentials and closed at high potentials but closed states at positive potentials are different from those at negative potentials. This is not explained by a simple electrostriction phenomenon. One cannot go from a positive to a negative closed state without reopening the channel (Colombini, 1986). More detailed information on the molecular basis for gating in VDAC is available in the publications of Song et al. (1998a,b).

There are two ways to measure the voltage-dependence parameters for VDAC: the use of distinct steps of voltage and the use of triangular voltage waves. When steps of voltage are applied to a multi-channel membrane, one typically begins with a voltage at which the channel is open (typically between 10 mV and -10mV) and then steps the voltage up to the desired value. The conductance is allowed to relax to a steady value and that value is assumed to be an equilibrium value reflecting the conductance through some closed channels and some open channels in dynamic equilibrium. This process is repeated until a voltage range is spanned from voltages where essentially all the channels are open to voltages where they are essentially all closed. The problem with this approach stems from the complexity of the gating of VDAC. There is only one high-conductance, anion-selective, open state and a large set of lower conducting and usually cation-preferring states called closed states. Some of these are readily accessible but others are only accessible after prolonged application of an elevated potential. The voltage step approach results in VDAC occupying a different population of states at low and high voltages. This becomes evident when single channels are analyzed.

The use of triangular voltages is not the ideal method but the best available method. It takes advantage of the large difference in rates between the voltage-dependent closing process (msec to minutes) and the largely voltage-independent opening process (submillisecond). It also probes the system in the time scale of 1 min (for channel reopening) by applying triangular waves or 2-5 mHz. Thus one is probing the voltage-dependence of rapidly-accessible closed states. (Physiologically, the other states are going to be important as well.) One analyzes the part of the recording where the channels are reopening so that the record is close to an equilibrium between rapidly-accessible

states. This approach yields results that are essentially independent of the speed of the triangular wave from 1 to 10 mHz. The results are fit to a Boltzmann distribution following the fundamental work of Hodgkin and Huxley (1952).

2. Simple protocol for observing basic properties of VDAC:

a) Reagents:

Chamber solution: 1.0 M KCl, 1 mM $MgCl_2$, 5 mM HEPES pH 7.5

b) Protocol:

Generate a planar membrane using the desired lipid mixture in the presence of the chamber solution. Begin recording the current through the membrane. Insert VDAC as described in III.C.2 with an applied voltage of 10 mV. If one channel inserts (4 nS conductance), step the voltage to 40 mV and you should observe a 4-fold increase in current (Ohmic behavior). With time the current should tend to drop to a lower value, about 40% of the original. The current should move stepwise between discrete levels. There should be one high-current level corresponding to a conductance of 4 nS (the open state) and a variety of low-conducting levels (closed states). When the voltage is returned to 10 mV, the channel should reopen quickly and return to its original value. Usually this opening transition is only detected at high time resolution (a few msec). The closing rate at 40 mV is in the sec time range. The closing rate increases as the applied voltage is increased. Voltages greater that 70 mV are quite likely to result in membrane breakdown and this likelihood increases with the magnitude of the applied voltage. If the channel is held closed for several minutes, the reopening rate should be much slower and the application of an opposite potential may be needed. VDAC develops a memory of the applied voltage that becomes erased when the channel reopens. This voltage

gating should be detected at both positive and negative applied potentials. During the experiment, more channels may insert making the recordings more complex.

If many channels insert, the same voltage step protocol can be used but one sees the current changes resulting from the action of many channels. Figure 5 shows an experiment in which 5 channels inserted. A group of 3 channel inserted in unison (first current step then two single channels inserted. When the voltage was increased to 40 mV, there are clearly 5 levels of current drop corresponding to the closure of 5 channels. When the voltage was returned to 10 mV, the current instantly returned to the level it was prior to the transition to 40 mV because all 5 channels opened rapidly.

If hundreds of channels have inserted, the current changes will look like exponentials as the population is large enough to smooth out the stochastic variation.

IV. Summary

Quantitative measurements of the permeability of the mitochondrial outer membrane to metabolites allows one to assess the ability of mitochondria to exchange metabolites with the cytosol of the cell. This can be used to determine the influence of treatments or conditions that alter this permeability to regulate cellular metabolic rate or control the initiation of the apoptotic process by closing VDAC. By comparing effects on pure VDAC with those in the outer membrane one can discover the existence of endogenous factors that influence VDAC's response. Naturally the isolated mitochondrion lacks any cytosolic influences but cytosolic factors could be added back to determine their mode of action.

V. Acknowledgments

This work was supported by NIH grants: AI49388, NS42319, NS42025

VI. References

Chiu, C., Su, S., and Russel, P.J. (1967). Adenylate kinase from baker's yeast: I Purification and intracellular location. *Biochim. Biophys. Acta* 132, 361-369.

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

Colombini, M. (1986). Voltage gating in VDAC: toward a molecular mechanism. In: Ion Channel Reconstitution (Miller, C., ed.) Section 4, Chapter 10. Plenum Press, New York.

Colombini, M. (1987). Characterization of channels isolated from plant mitochondria. *Methods Enzymol*. 148, 465-475.

Colombini, M. (1989). Voltage gating in the mitochondrial channel, VDAC. *J. Membr. Biol.* 111, 103-111.

Colombini, M. VDAC: the channel at the interface between mitochondria and the cytosol. (2004). *Molec. Cell. Biochem.* 256, 107-115.

Elkeles, A., Breiman, A., and Zizi, M. (1997). Functional differences among wheat voltage-dependent anion channel (VDAC) isoforms expressed in yeast. Indication for the presence of a novel VDAC-modulating protein? *J. Biol. Chem.* 272, 6252-6260.

Freitag, H., R.Benz, and W.Neupert. (1983). Isolation and properties of the porin of the outer mitochondrial membrane from Neurospora crassa. *Methods Enzymol.* 97, 286-294.

Gincel. D, Silberberg, S.D., and Shoshan-Barmatz, V. (2000). Modulation of the Voltage-Dependent Anion Channel (VDAC) by glutamate. *J. Bioenerg. Biomembr.* 32, 571-583.

Hodgkin, A.L., and Huxley, A.F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (London)* 117, 500-544.

Jonas, E.A., Hickman, J.A., Hardwick, J.M., and Kaczmarek, L.K. (2005). Exposure to hypoxia rapidly induces mitochondrial channel activity within a living synapse. *J. Biol. Chem.* 280, 4491-4497.

Komarov, A.G., Deng, D., Craigen, W.J. and Colombini, M. (2005). New insights into the mechanism of permeation through large channels. *Biophys. J.*, 89, 3950-3959

Lee, A., and Colombini, M. (1997). A unique method for determining the permeability of the mitochondrial outer membrane. *Methods Cell Sci.* 19, 71-81.

Lee, A., Xu, X., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998). The role of yeast VDAC genes on the permeability of the mitochondrial outer membrane. *J. Membr. Biol.* 161, 173-181.

Lee, A., Zizi, M., and Colombini, M. (1994). β-NADH decreases the permeability of the mitochondrial outer membrane to ADP by a factor of 6. *J. Biol. Chem.* 269, 30974-30980.

Lemasters, J.J., and Holmuhamedov, E. (2006). Voltage-dependent anion channel (VDAC) as mitochondrial governator--thinking outside the box. *Biochim Biophys Acta.* 1762:181-190.

Mannella, C.A., Forte, M., and Colombini, M. (1992). Toward the molecular structure of the mitochondrial channel, VDAC. *J. Bioenerg. Biomembr.* 24, 7-19.

Montal, M., and P.Mueller. (1972). Formation of bimolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. USA.* 69, 3561-3566.

Pfaff, E., Heldt, H.W., and Klingenberg, M. (1969). Adenine nucleotide translocation of mitochondria. Kinetics of the adenine nucleotide exchange. *Eur. J. Biochem.* 10, 484-493.

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

Song, J., Midson, C., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998a). The sensor regions of VDAC are translocated from within the membrane to the surface during the gating processes. *Biophys. J.* 74, 2926-2944.

Song, J., Midson, C., Blachly-Dyson, E., Forte, M., and Colombini, M. (1998b). The topology of VDAC as probed by biotin modification. *J. Biol. Chem.* 273, 24406-24413.

Su, S., and Russel, P.J. (1968). Adenylate kinase from baker's yeast. *J. Biol. Chem.* 243, 3826-3833.

Tedeschi, H., Kinnally, K.W., and Mannella, C.A. (1989). Properties of channels in the mitochondrial outer membrane. *J. Bioenerg. Biomembr.* 21, 451-459.

Werkheiser, W.C., and Bartley, W. (1957) The study of the steady-state concentration of internal solutes of mitochondria by rapid centrifugal transfer to a fixation medium. *Biochem. J.* 66, 79-91.

Wunder, U.R., and Colombini, M. (1991). Patch-clamping in liposomes containing whole mitochondrial membranes. *J. Membr. Biol.* 123, 83-91.

Xu, X., and Colombini, M. (1996). Self-catalyzed insertion of proteins into phospholipid membranes. *J. Biol. Chem.* 271, 23675-23682.

Zizi, M., Byrd, C., Boxus, R., and Colombini, M. (1998). The voltage-gating process of the VDAC channel is sensitive to ion flow. *Biophys. J.* 75, 704-713.

Zizi, M., Thomas, L., Blachly-Dyson, E., Forte, M., and Colombini, M. (1995). Oriented channel insertion reveals the motion of a transmembrane beta strand during voltage gating of VDAC. *J. Membr. Biol.* 144, 121-129.

V. Figure Legends:

Figure 1. Illustration of the processing of respiration data to obtain a quantitative measure of the permeability of the mitochondrial outer membrane. The left panel shows a drawing of a typical mitochondrial respiration curve obtained after the addition of ADP. The data needs to be collected directly via an A/D converter with at least 10 bit accuracy. The state 4 respiration rate is subtracted from the recording by fitting a straight line to the state 4 recording (use the asymptote to the end part of the data) and subtracting this from all the data. Only the record after ADP addition is used for further calculations. Using the standard P/O ratio, the oxygen concentration is converted to the ADP concentration (right panel). Each state3/state4 transition takes approximately 5 min. See text for further explanations.

Figure 2. Illustration of the processing of NADH oxidation data to obtain a quantitative measure of the permeability of the mitochondrial outer membrane. The panel on the left shows an illustration of typical recordings of NADH oxidation by intact and hypotonically-shocked mitochondria obtained from the yeast, *S. cerevisiae*. The absorbance at 340 nm was converted to [NADH] by using the extinction coefficient (6.22×10^3 molar absorbancy). Rates of NADH oxidation were obtained by determining the tangents at each point from the data in the left panel (see text). These rates were plotted as a function of the [NADH]. For each point on the curve for intact mitochondria one can obtain the medium [NADH] (subscript "o") by dropping a perpendicular and the intermembrane space [NADH] (subscript "i") by finding the point on the shocked mitochondria curve

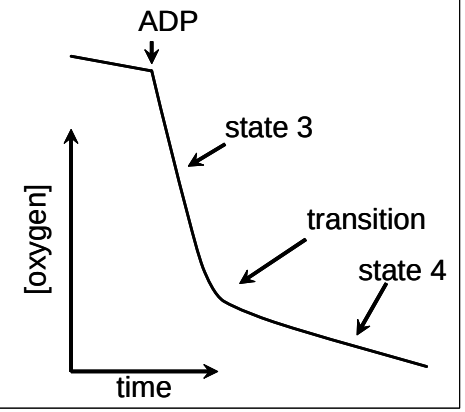
and dropping a perpendicular from that point. This determination was not obtained graphically (as explained in the text) but it is hoped that the illustration with clarify how the information can be obtained. Each experiment takes 10-30 min depending on the permeability of the outer membrane.

Figure 3. Sample results of experiments to measure the permeability of the mitochondrial outer membrane by measuring adenylate kinase activity. Mitochondria were isolated from *S. cerevisiae* cells, both wild type (VDAC containing) and lacking VDAC1. The experiment was run as described in the text.

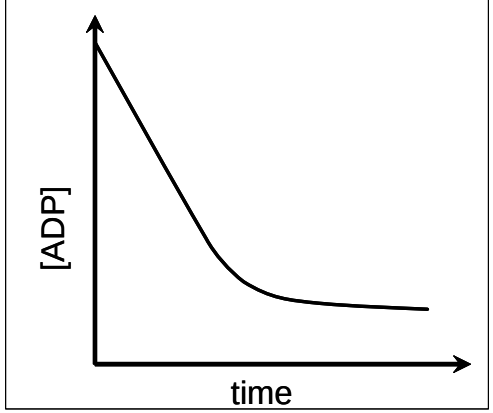
Figure 4. Experimental set-up for recording the conductance of VDAC channels reconstituted into planar membranes. The Teflon chamber consists of 2 hemi-chambers separated by a thin partition containing the small hole (0.1 mm in diameter) across which a phospholipid membrane is formed. The aqueous solutions on the two sides are shown after both levels have been raised and the membrane would have been formed. The bottom of each hemi-chamber has a small well containing the Teflon-coated stir bar for mixing the solutions. A DC powered geared motor is used to rotate a magnetic bar below the chamber and this bar rotates both stir bars simultaneously. The rate of mixing is varied by varying the voltage applied to the motor. The electrodes are made from calomel and contain salt bridges. Syringes are used to control the level of the solution. Only one syringe is illustrated for clarity. No needles are used. Rather Tygon tubing is used to connect the syringe to the solution. The electronics illustrated is that of a high-quality operational amplifier in the inverted mode. One can certainly use a commercial amplifier instead. The voltage is clamped and the current measured.

The compartment held at virtual ground by the amplifier is labeled “trans” and the compartment whose voltage is controlled by the operator is labeled “cis”.

Figure 5. An example of a recording of the insertion of 5 VDAC channels and their voltage-dependent closure. These channels were isolated from *N. crassa* mitochondria. *N. crassa* VDAC channels often insert as groups of 3 channels (see first insertion). Addition of the VDAC-containing sample to the cis compartment while stirring produces a noisy record. When the stirring is stopped the noise is no longer there. During the addition and channel-insertion period, the applied voltage was 10 mV. At the indicated time point, the voltage was raised to 40 mV. After all the channels closed, the voltage was reduced to 10 mV.

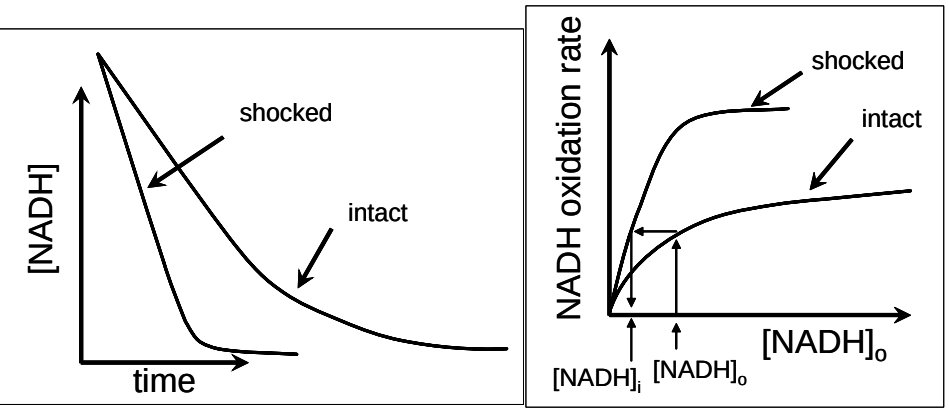


original record



rate of ADP consumption

Figure 1



original records

rates of oxidation

Figure 2

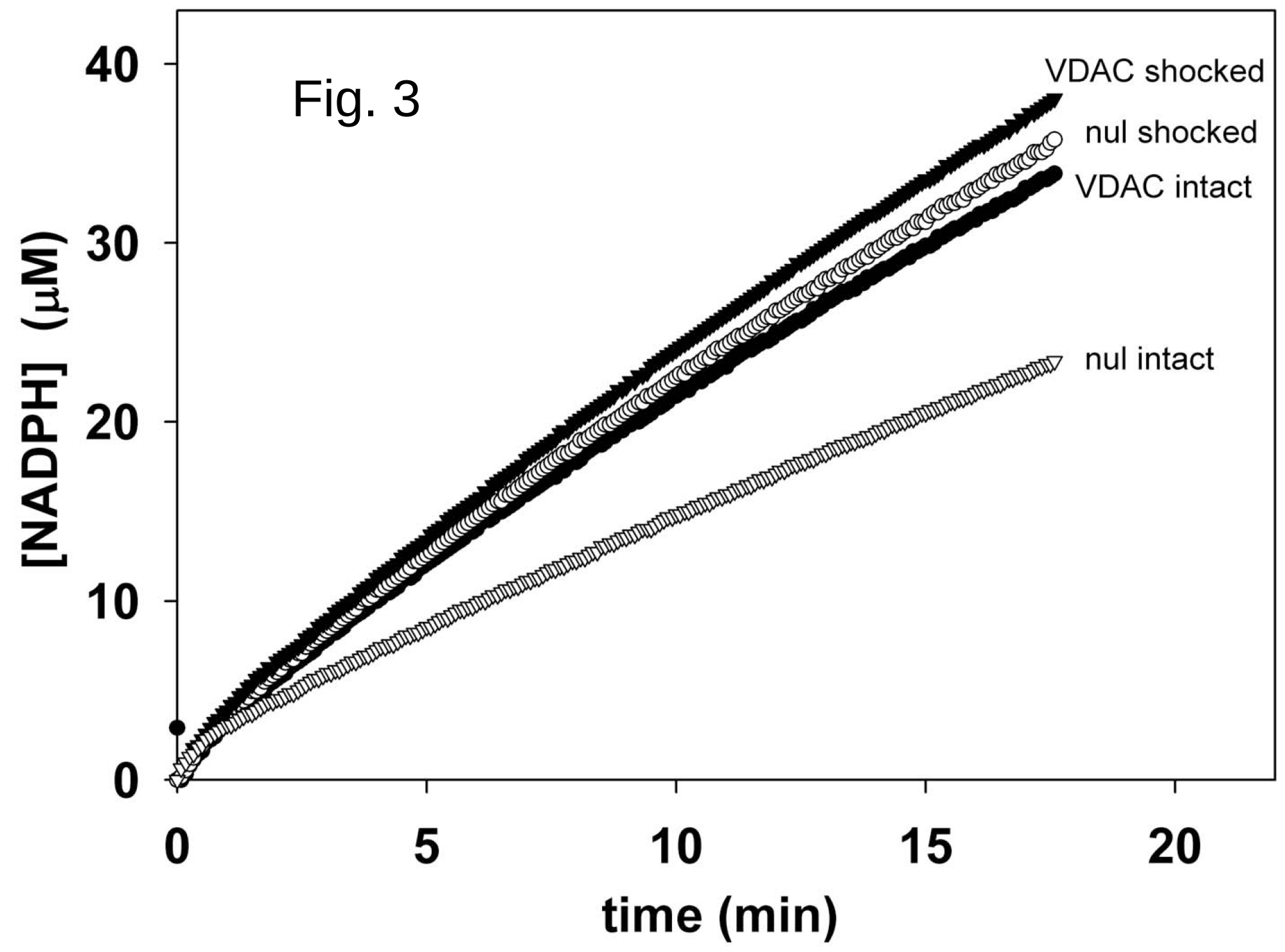


Fig. 3
VDAC shocked
nul shocked
VDAC intact
nul intact
[NADPH] (µM)
0
10
20
30
40
0
5
10
15
20
time (min)

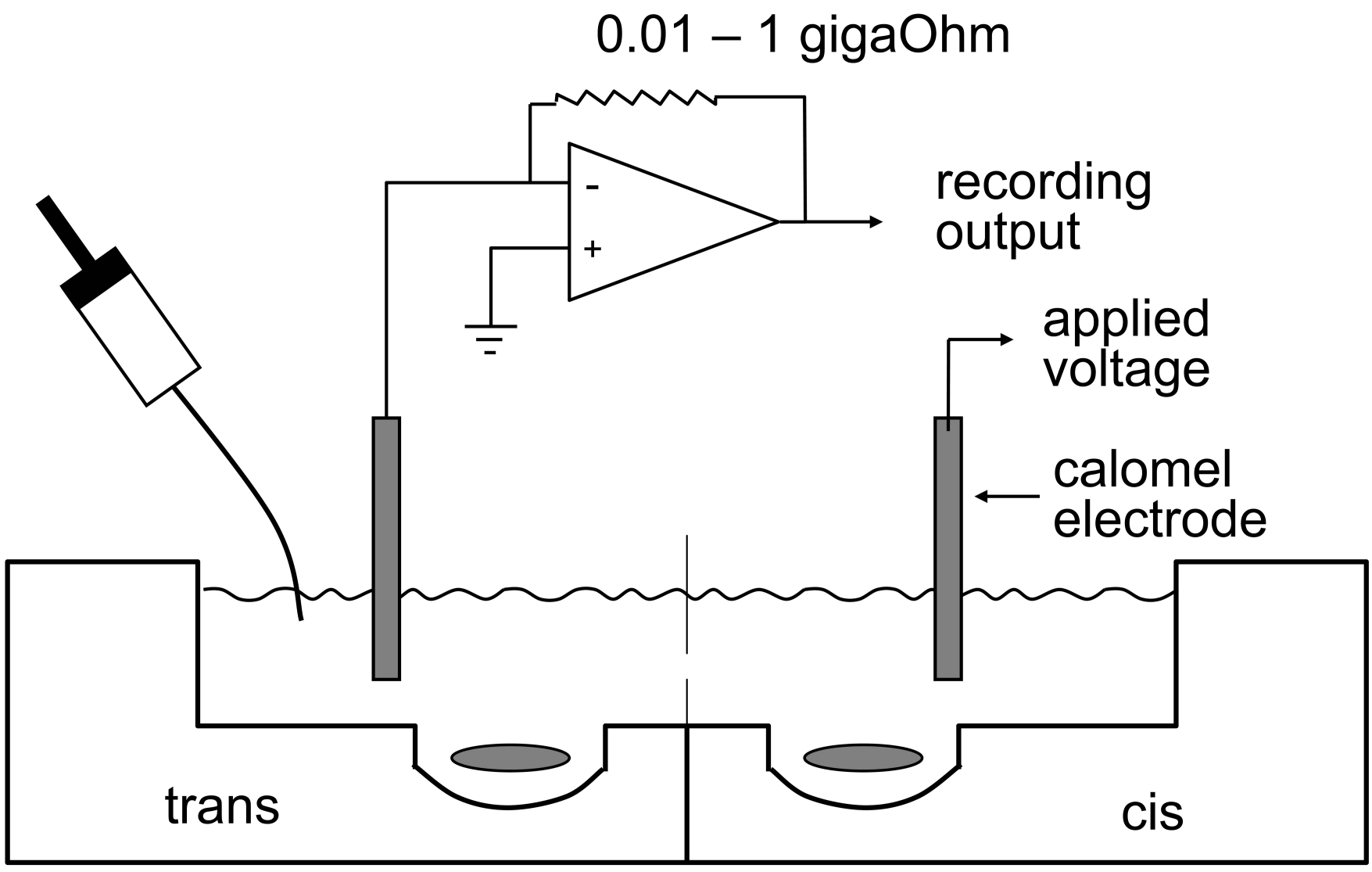

0.01 – 1 gigaOhm
-
+
recording
output
applied
voltage
calomel
electrode
trans
cis

Fig. 4

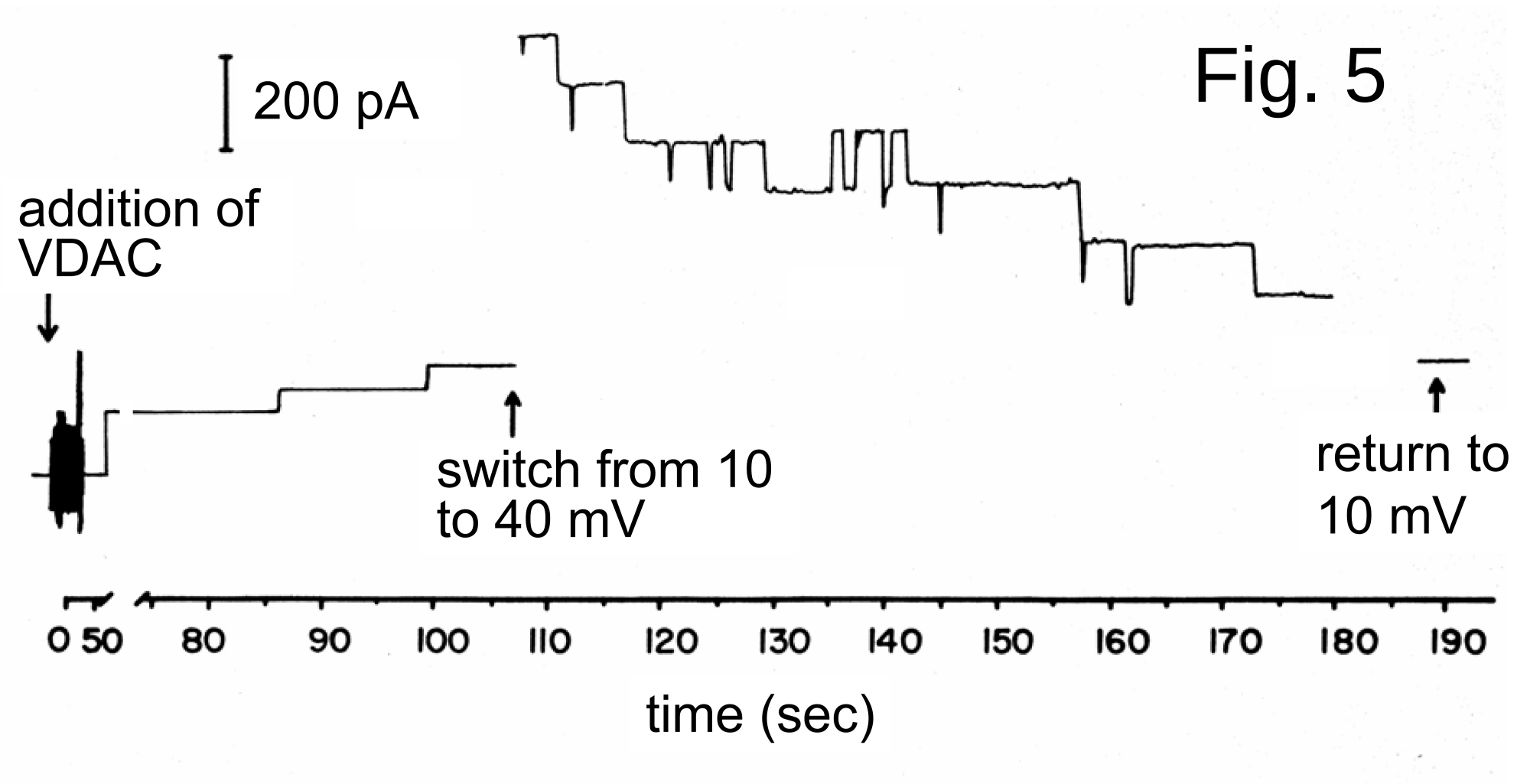


Fig. 5
200 pA
addition of
VDAC
switch from 10
to 40 mV
return to
10 mV
0 50 80 90 100 110 120 130 140 150 160 170 180 190
time (sec)