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The mitochondrial outer membrane channel, VDAC, is modulated by a soluble protein

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The mitochondrial outer membrane channel, VDAC, serves as the primary permeability pathway for metabolite flux between cytoplasmic and mitochondrial compartments. VDAC can occupy several conformational states differing in ion conductivity. Small transmembrane potentials cause transitions from open- to closed-channel conformations. A soluble mitochondrial protein enhances the channel's response to voltage by increasing the rate of channel closing; inducing the occupation of lower conductance states; and decreasing the rate of channel reopening. This protein modulator acts at very low concentrations and its role in the cell may be to regulate the permeability of the mitochondrial outer membrane by inducing channel closure.

Ion channel; Voltage-dependent channel; Mitochondria; Regulation; Mitochondrial membrane; (*Neurospora crassa*)

1. INTRODUCTION

A large diameter channel, VDAC (or mitochondrial porin), provides the primary permeability pathway through the mitochondrial outer membrane for small molecules necessary for mitochondrial respiration and metabolism. The functional properties of VDAC have been studied primarily by reconstitution of the channel into planar phospholipid membranes. A highly conserved and important property of the channel is voltage dependence. The channel is found in a high conductance or open state in the absence of a transmembrane potential. As the transmembrane potential is raised (both positive and negative), the probability that the channel will assume a reduced conductance or 'closed' state increases [1,2]. The channel does not become totally nonconductive. Channel conductivity can also be regulated by synthetic polyanions [3–6] which dramatically increase the steepness of the voltage dependence. This paper reports the discovery of a protein in the mitochondrial subcellular fraction, referred to as the modulator, which modifies the voltage-dependent regulation of VDAC.

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2. MATERIALS AND METHODS

Both VDAC and the modulator protein were obtained from the mitochondrial fraction of a wall-less mutant of *Neurospora crassa* (ATCC no.32360). The mitochondrial membranes, prepared according to Mannella [7] with the modifications of Dill et al. [8], were used as the source of VDAC. Modulator protein was obtained from a washed mitochondrial fraction prepared according to Mannella [7], except that the mitochondria were pelleted at 11000 × *g* (20 min). Mitochondria were sonicated in 3 ml of buffer (10 mM Tris-HCl, 10 mM KCl, pH 7.5) using a bath sonicator. Mitochondrial membranes were pelleted at 150000 × g_{max} (Beckman L3-50, 50-Ti rotor, 30 min). The modulator activity was found in the supernatant. The modulator was also obtained from mitochondria purified on percoll gradients using the procedure for potato mitochondria [9].

Experiments were conducted with VDAC reconstituted into planar phospholipid membranes made by the monolayer method of Montal and Mueller [10] and studied under voltage-clamp conditions as described in Schein et al. [11]. Membranes were prepared from either a synthetic lipid, diphytanoyl phosphatidylcholine (DPPC, Avanti Biochemicals, Bir-

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mingham, AL), or a combination of DPPC and partially purified [12] soybean phospholipids (SP, type II-S phosphatidylcholine, Sigma, St. Louis, MO). VDAC solubilized in 1% Triton X-100 was added to one aqueous phase (*cis* compartment) and spontaneous insertion of channels was monitored at 10 mV applied potential. In all experiments the aqueous phase consisted of 1 M KCl and 5 mM $CaCl_2$. The sign of the potential refers to the *cis* compartment, the *trans* compartment is virtual ground.

3. RESULTS AND DISCUSSION

A soluble protein found in the mitochondrial fraction of *Neurospora crassa* cells modulated the voltage-dependent behavior of VDAC channels reconstituted into planar phospholipid membranes in a highly reproducible and dose-dependent manner. The modulator protein increased the sensitivity of VDAC to voltage in three ways: it increased the rate of channel closure; decreased the rate of opening; and induced the channels to assume lower conductance closed states.

The addition of a small aliquot of the modulator preparation (crude or partially purified) to the aqueous compartment dramatically increased the rate of channel closure at 60 mV (fig.1). Prior to modulator addition channel closure was a slow process with an apparent time constant of approx. 22 s (fig.1A) [Higher potentials were required to close the channels in DPPC membranes than those required with soybean phospholipids (SP). The use of the DPPC made it easier to monitor and quantitate the action of the modulator. The effects of the modulator were also observed with SP membranes.]. Addition of the modulator to the *trans* side effected an initial reduction of the apparent time constant to 5 s (fig.1B) and after a few minutes to <2 s (fig.1C). The addition of pronase to the chamber on the same side as the modulator rapidly reversed the effect of the modulator (fig.1D).

VDAC exhibits symmetric behavior, that is, the channels close at about the same rate and to about the same extent regardless of the sign of the applied potential. However, the modulator-enhanced closure required that the applied potential be negative on the side of the membrane where the modulator preparation was added. In fig.1, modulator was added to the *trans* side and positive potentials on the *cis* side, i.e. negative *trans*, were needed to observe its effect. The requirement for a negative potential on the modulator side held

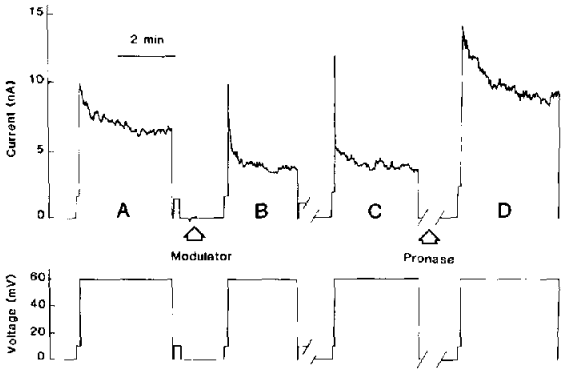

Fig.1. The modulator increased the rate of VDAC closure. Records of the current passing through a DPPC membrane containing 40–60 channels prior to (A) and following (B,C) the addition of a modulator-containing sample to the *trans* compartment (30 μg protein/ml, final conc. of crude prep.). Pronase (36 μg/ml, final conc.) was added to the *trans* compartment (D). The increasing current during the experiment was due to slow insertion of channels.

whether the modulator was added to either the *cis* or *trans* compartments. Modulator addition to both sides resulted in enhanced closure with both positive and negative potentials. Thus the modulator protein probably interacts with either exposed face of the channel.

We also observed that, in the presence of modulator, the steady-state conductance after potential-induced channel closure was always less than the control. In fig.1, the closed-channel conductance of the control (fig.1A) was 65% of the open-channel conductance versus 33% for the treated (fig.1C). The modulator could be acting as a channel blocker, inducing more channels to close, or encouraging individual channels to assume a lower conductance closed state. It will be shown that the latter two possibilities are involved.

The modulator significantly slowed the opening kinetics of VDAC. Closed VDAC channels ordinarily reopen with a rate of microseconds to milliseconds when the transmembrane voltage is reduced to 10 mV or less [11,13,14]. However, in the presence of the modulator, channels were slower to open and some remained closed for minutes. In fig.2, modulator was added to the *cis* side of a membrane containing two channels. Once the channels were closed by applying −50 mV, neither of the two channels reopened when the transmembrane potential was returned to −10 mV

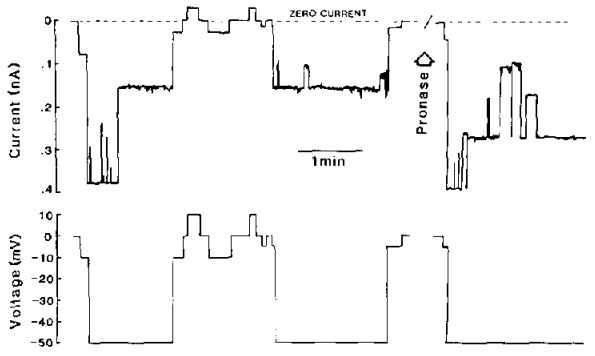

Fig.2. The modulator decreased the rate of VDAC opening. The SP:DPPC (50:50, v/v) membrane contained 2 channels and the modulator (18 μg protein/ml, of crude prep.) was present in the *cis* compartment 4.5 min prior to the beginning of the record. The channels remained closed during the time break (6 min). Channels reopened 1 min after pronase addition (20 μg/ml, final conc. in *cis* compartment).

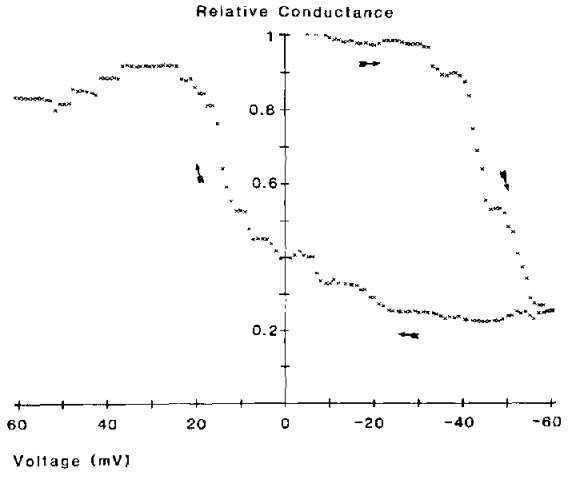

Fig.3. The modulatory delayed channel opening. There were approx. 10 channels in the DPPC membrane and the modulator (15 μg protein/ml, of crude prep.) was present in the *cis* compartment. The membrane was subjected to successive triangular voltage waves. The current record from one wave (2 mHz) was used to calculate conductance. All channels were open and thus maximally conductive (normalized to 1) at 0 mV where the record begins. Arrows indicate the time course of the voltage wave. The channel closure at high positive potentials is the result of normal voltage-dependent closure of VDAC in DPPC, see text.

or even to positive voltages. However, the addition of pronase resulted in channel reopening. In some experiments, the application of a small (10 mV) positive potential would induce closed channels to reopen.

Similar modulator effects on opening kinetics were observed with membranes containing many channels. Fig.3 shows a record of voltage vs conductance (as a measure of the number of open channels) of a modulator-treated, multichannel membrane. When a 2 mHz triangular voltage wave (0.48 mV/s) was applied, the observed conductance change showed marked path dependence demonstrating extreme hysteresis (arrows indicate the temporal progress of the experiment). The applied voltage began at zero and decreased to -60 mV resulting in a conductance decrease as the channels closed (upper trace), mostly between -40 and -60 mV. The voltage was then increased from -60 mV to $+60$ mV (lower trace) but few channels reopened before 0 mV was reached. A positive potential (10–20 mV) on the *cis* (modulator) side induced reopening of most closed channels. In contrast, in the absence of modulator all channels would have reopened when 0 mV was reached. That was indeed the case when the voltage wave progressed from $+60$ mV to 0 mV (not shown), because modulator was present only on the *cis* side. Thus at the beginning of this trace all channels were open. The hysteresis demonstrated in fig.3 indicates that the rate of change of the applied voltage was faster than VDAC's opening kinetics (This type of measurement of conductance vs voltage has always been determined with decreasing voltage because the opening kinetics are normally so rapid that an equilibrium state can be assumed when slow triangular waves are employed. See [1,3,15] for examples.). Therefore, the opening rates were at least 1000 × slower than they were prior to modulator addition [11,13,14].

The modulator is likely to be a protein. Evidence for this comes from sensitivity of the modulator to protease action: i.e. trypsin and pronase. Modulator enhancement of the rate of VDAC-channel closure was eliminated in 5 min or less after the addition of pronase to the modulator-containing aqueous phase (figs 1D and 2). Trypsin eliminated the modulator activity at a slower rate (not shown). VDAC channels, inserted into bilayers, are unaffected by pronase or trypsin, either in our own control experiments or in the experiments of others [16,17].

The modulator is very potent, being active and detectable at nanomolar quantities. Modulator

preparations were partially purified by chromatography on Sephacryl S-300 gel filtration columns. The elution profile of the active fractions from the calibrated column suggested a molecular mass range of 84–92 kDa for the modulator protein. SDS-PAGE of active fractions showed the presence of several bands. From the amount of protein in an active fraction added to the reconstituted system and preliminary determination of the modulator's molecular mass, it was estimated that nanomolar quantities of the modulator were sufficient to observe a clear response. This high potency is more compatible with a substance that is designed to interact with the channel as opposed to a secondary, nonspecific effect. However, it has been reported that many polyanions (in the micromolar range) increased VDAC's voltage dependence. We do not view the modulator as another polyanion for the following reasons: (i) polypeptides with high concentrations of negative charge (e.g. pepsin, polyaspartate) require concentrations 3–4 orders of magnitude higher [6]; (ii) there are substantive differences between the action of polyanions and the modulator; (iii) preliminary experiments with ion-exchange columns indicated that the modulator is not highly charged; (iv) a variety of proteins (not highly charged) were tested for their effect on VDAC's behavior and no changes were seen (not shown).

To distinguish between the modulator acting by influencing the nature of the closed-channel state or by a block mechanism, we observed the effects of the modulator on membranes with only 1–3 channels incorporated. This allowed us to quantitate the conductance of individual closed states. In fig.4 three channels were present. The first −50 mV pulse resulted in the closure of one and sometimes two channels. Upon addition of modulator, the channels closed at a greatly enhanced rate. During the second voltage pulse, three large conductance drops were recorded followed by a smaller conductance drop. After a second modulator addition, three large and three small decrements were observed. We interpret these results to mean that each channel has undergone two separate transitions, a large followed by a small conductance drop, with the final closed-channel conductance averaging about 10% of the open-channel conductance.

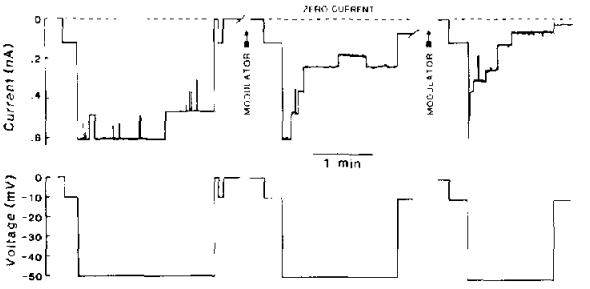

Fig.4. Records of the current passing through a DPPC membrane containing 3 channels. Modulator (15 μg protein/ml, final conc. of crude prep.) was added 6 min before application of the second (−) 50 mV pulse. A second modulator addition for a final concentration of 30 μg protein/ml was made less than 1 min prior to the third pulse.

We observed the behavior of single channels in many separate experiments (in the presence or absence of modulator) to determine if the second conductance drop to a lower conductance closed state was unique to VDAC interaction with the modulator. Histograms of conductance drops indicate that there is no significant difference between control and modulator treatment in terms of the type of drops recorded (fig.5A,B) [The broad diversity of transitions was mostly due to occupation by individual channels of multiple closed states [1,17,18]. As the open-channel conductance is quite uniform [15], differences between individual channels could only partially account for the diversity.]. However, the frequency of secondary smaller events (0.3–1.5 nS) was greater with modulator-treated channels indicating that the modulator is facilitating this second transition. These results are inconsistent with a blocking mechanism.

Our observations indicate that the modulator makes the channel more sensitive to voltage. All of the observed changes in VDAC's behavior due to modulator addition can be mimicked (at least qualitatively) by applying high enough potentials and/or for long enough time. Although it is likely that the modulator can induce behavioral/conformational states in VDAC that are unique, there is currently no clear evidence for these.

At present, there is much interest in a possible role for the outer membrane in regulating mitochondrial function. As VDAC serves as the conduit for molecular traffic across the outer

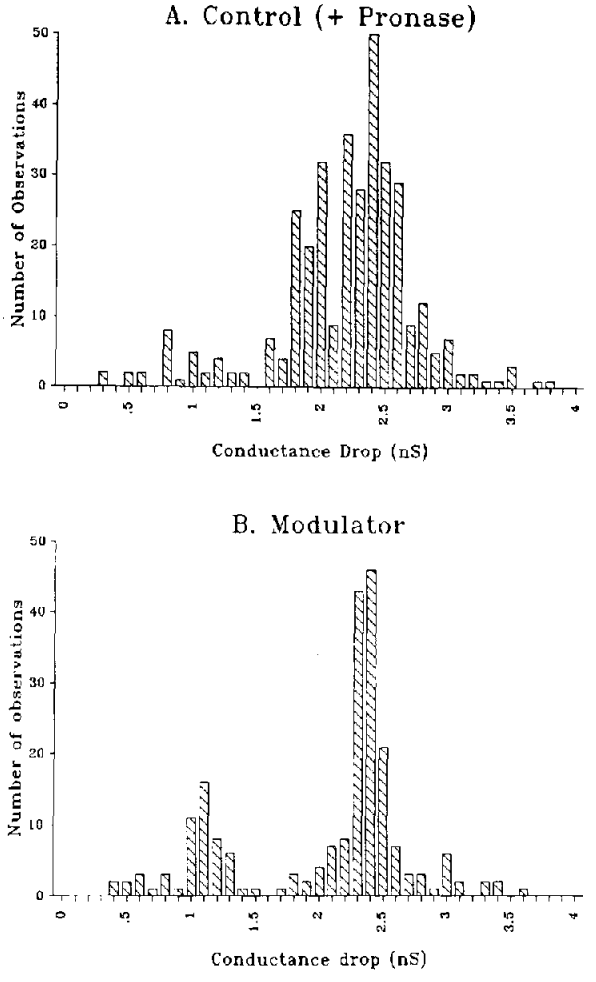

Fig.5. Histogram of drops in channel conductance recorded from DPPC or SP:DPPC (50:50) membranes containing 1 to 3 channels (records similar to fig.4). The aqueous phase for control (A) membranes contained pronase (20–200 μg/ml, final concentration) to destroy any modulator that might be present in VDAC preps. Mean conductance for large decrement in conductance (1.6–3.0 nS) was 2.3 nS for control and 2.4 nS for modulator-treated channels, while for the small decrements (0.5–1.5), the mean was 0.9 nS for control and 1.0 nS for treated.

membrane, it is a likely candidate for a role in regulating, for example, the rate of mitochondrial respiration by controlling the flux of metabolites. Conductance states of VDAC can be controlled by voltage. This voltage control could be achieved in vivo by a non-dissipative potential-generating process (Donnan or surface potential), or perhaps by location of channels in areas of close apposition of inner and outer membranes [19]. Voltage control is greatly augmented by the potent modulator described here. We have preliminary evidence that this modulation may not be unique to *Neurospora*. Extracts from yeast mitochondrial preparations contain a factor which exerts similar effects on *N. crassa* VDAC.

Continued research on the modulator will take several directions. It is important to elucidate further the molecular mechanism of action of the modulator, i.e. does the modulator act on the voltage sensor as has been suggested for polyanions [3]. Purification of the modulator and determination of its precise subcellular location will be important steps toward ascertaining the physiological significance of the modulator.

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