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Research Paper

A Soluble Mitochondrial Protein Increases the Voltage Dependence of the Mitochondrial Channel, VDAC

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A soluble protein isolated from mitochondria has been found to modulate the voltage-dependent properties of the mitochondrial outer membrane channel, VDAC. This protein, called the VDAC modulator, was first found in *Neurospora crassa* and then discovered in species from other eukaryotic kingdoms. The modulator-containing fraction (at a crude protein concentration of 20 μg/ml) increases the voltage dependence of VDAC channels over 2–3-fold. At higher protein concentrations (50–100 μg/ml), some channels seem to remain in a closed state or be blocked while others display the higher voltage dependence and are able to close at low membrane potentials. By increasing the steepness of the voltage-dependent properties of VDAC channels, this modulator may serve as an amplifier *in vivo* to increase the sensitivity of the channels in response to changes in the cell's microenvironment, and consequently, regulate the metabolic flux across the outer mitochondrial membrane by controlling the gating of VDAC channels.

KEY WORDS: Ion channel; voltage-dependent channel; mitochondrial outer membrane; modulating protein; VDAC; channel regulation.

INTRODUCTION

Most studies on mitochondrial metabolism have focused on the enzymes in the inner membrane and the matrix compartment. The mitochondrial outer membrane was considered to be a leaky membrane, and to play no significant role in mitochondrial functions. The discovery that the outer membrane contains a voltage-dependent anion-selective channel (VDAC) (Schein *et al.*, 1976; Colombini, 1979) and this channel is the major permeability pathway through the outer membrane has resulted in the suspicion that the outer membrane may play an important role in mitochondrial metabolism. The finding that hexokinase binds to mitochondria via VDAC channels (Linden *et al.*, 1982b; Fiek *et al.*, 1982; Krause *et al.*, 1986), the possible localization of VDAC in contact sites (Adams *et al.*, 1989), and the discovery of the VDAC modulator (Holden and Colombini, 1988; Liu and Colombini, 1991) have further heightened the perceived importance of the VDAC channel and the outer membrane.

It has been known that voltage-dependent ion channels play an important role in many physiological processes, such as production and transduction of electrical signals in excitable cells. The VDAC channel is a 30-kDa polypeptide, constituting more than 60% of the outer membane proteins in some organisms (Mannella, 1982). When incorporated into phospholipid bilayer membranes, VDAC channels exist in a highly conductive open state at zero and low potentials, but the channels are induced into a low-conducting closed state at high transmembrane potentials. In the closed state, VDAC has not only a reduced pore size and pore volume (Colombini *et al.*, 1987; Zimmerberg and Parsegian, 1986), but also a reduced permeability and reversed selectivity (Colombini, 1980; Benz *et al.*, 1990; Colombini, 1989; Zhang and Colombini, 1990).

The transmembrane potential determines the

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probability of a voltage-dependent channel existing in a particular conducting state, and consequently determines the membrane permeability. The steeper the voltage dependence of a channel, the more responsive is the cell or organelle to small changes in the membrane potential, which would provide a sensitive way to mediate the cell's reactions to a changing micro-environment.

The voltage dependence of VDAC channels is normally an *e*-fold change for 5–7 mV in soybean phospholipid membranes (Mangan and Colombini, 1987), but this property can be greatly increased by synthetic polyanions, such as dextran sulfate (Mangan and Colombini, 1987) and König's polyanion (Colombini *et al.*, 1987). The result is an ultra-steep voltage dependence and an *e*-fold change for only 0.5 mV.

A soluble mitochondrial protein, called the VDAC modulator, was first found to regulate the gating behavior of VDAC channels in *N. crassa* (Holden and Colombini, 1988). Proteins with similar modulating functions were also discovered in mammals and plants (Liu and Colombini, 1991). In this paper, we report that this modulating protein increases the voltage dependence of VDAC, and induces the channel to enter a less conducting, less permeable closed state.

MATERIALS AND METHODS

Isolation of VDAC Channels

Mitochondrial outer membranes from a wall-less mutant of *N. crassa* (ATCC 32360) were isolated as described by Mannella (1982) with minor modifications. The mitochondrial outer membrane pellet was suspended in 10 mM KCl, 10 mM MOPS, and 1 mM EDTA, pH 7.2, supplemented with dimethylsulfoxide (DMSO) to 15% (vol/vol), and stored at −20°C or −70°C. Triton X-100 (final concentration 1%) was used to extract and solubilize VDAC channels from the outer membranes prior to the experiments.

Preparation of the Modulator-Containing Fraction

Mitochondria from *N. crassa* were isolated and washed as previously described (Mannella, 1982). The mitochondria were lysed in a hypotonic solution (10 mM MOPS, 1 mM EDTA, pH 7.2) on an ice bath to selectively break the outer membrane. The following protease inhibitors were added: 1 μg/ml pepstatin, 10 μg/ml chymostatin (1 mg/ml in DMSO), 1 μg/ml leupeptin, 4 μg/ml aprotinin, and 20 μM phenylmethylsulfonyl fluoride (100 mM in isopropanol). The lysed mitochondria were centrifuged at 12,000 × *g* for 20 min. The supernant was used as the modulator-containing extract and concentrated by using Amicon's Centriprep-10 (which has a molecular weight cut off of 10,000). The modulator-containing extract was recentrifuged at 100,000 × *g* to remove membranes and particles, stored at −80°C, and used as the modulator-containing fraction.

Generation of Planar Phospholipid Bilayers

All experiments were performed on planar phospholipid bilayer membranes which were generated by the monolayer method (Montal and Mueller, 1972) as previously described (Schein *et al.*, 1976; Colombini, 1987b). A synthetic lipid, diphytanoyl phosphatidylcholine (DPPC, Avanti Biochemicals, Birmingham, Alabama), was used to make the membranes. This lipid increased V_0 and made it easier to observe the effects of the modulator. A Saran partition with a 0.1-mm-diameter hole separated two compartments (labeled cis- and trans-sides). 1 M KCl, 5 mM $CaCl_2$, and 10 mM MOPS, pH 7.0, were used as the aqueous phases. Triton X-100 solubilized VDAC channels (3–5 μl) were added to the cis-side of the membranes. VDAC channels spontaneously inserted into the bilayer membranes.

Analysis of Voltage Dependence of VDAC Channels

The voltage-dependent properties of VDAC channels were studied under voltage-clamp conditions as previously described (Schein *et al.*, 1976; Colombini, 1987). Different voltages were applied to a membrane containing multiple VDAC channels. For each applied voltage, the resulting steady-state current was recorded and the corresponding conductance values were calculated.

By assuming the gating process of VDAC to be a two-state process, the probability of finding a channel in the open state can be fitted to the Boltzmann distribution (Ehrenstein *et al.*, 1970; Schein *et al.*, 1976) as follow:

$$\ln[(G - G_{min})/(G_{max} - G)] = (-nFV + nFV_0)/RT \quad (1)$$

where nFV and nFV_0 are the voltage-dependent and the voltage-independent energy differences between the open and the closed states. G, G_{min}, and G_{max} are the conductance at any voltage, the minimum conductance (all channels closed), and the maximum

conductance (all channels open) respectively, F, R, and T are the Faraday constant, the gas constant, and the absolute temperature, respectively, and n is a measure of the steepness of the channel's voltage dependence and can be interpreted as the minimum effective valency of the voltage sensor. The higher the n, the steeper the voltage dependence. If n were zero, the channel would not be voltage dependent. The quantity V_0 is the voltage at which one-half of the channels are closed or open, and $[(G - G_{min})/(G_{max} - G)]$ is the ratio of the number of open channels to the number of closed channels. By plotting $\ln[(G - G_{min})/(G_{max} - G)]$ vs. V, we obtained the values of n and V_0 (n obtained from the slope and V_0 from the voltage-axis intercept of the straight line). The values in the text are reported as means $\pm$ S.D.

RESULTS

When inserted into the planar phospholipid membranes, the mitochondrial outer membrane channel, VDAC, is voltage dependent, being open at zero and low membrane potentials and closed at high potentials. In all experiments, VDAC channels were inserted into the DPPC membranes bathed by the same aqueous medium (1 M KCl, 5 mM $CaCl_2$, 10 mM MOPS, pH 7.0) on both sides. After a stable multichannel membrane was formed, steps of different voltage were applied to the membranes. At high enough potentials, VDAC channels began to close and the current decreased with time until a steady state was achieved. The steady-state currents were measured and used to calculate the channel conductance for each applied voltage. Triangular waves were not used to assess the voltage dependence because the VDAC modulator greatly reduces VDAC's opening kinetics, resulting in hysteresis in the current record when triangular waves were applied (Holden and Colombini, 1988).

Figure 1 shows the effect of the VDAC modulator on the voltage dependence of the current flowing through a multichannel membrane. VDAC channels were first allowed to fully open at a low membrane potential, usually -10 mV. Then, by increasing the applied potential, we obtained time-dependent current decreases whose rate and magnitude depended on the applied voltage. The current instantaneously increased as the voltage drove ions through the channels and then decayed as the channels closed. In the figure, current flowing through the membranes

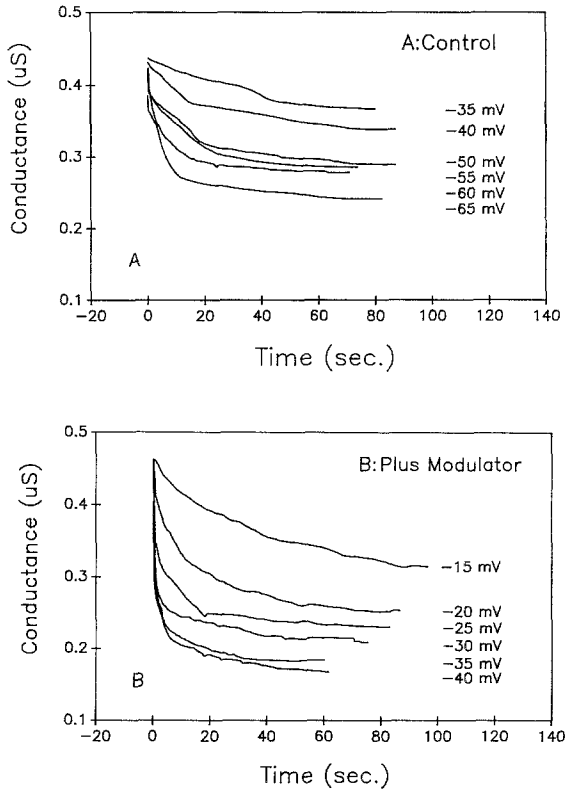

Fig. 1. Effect of the modulator on voltage-dependent closure of VDAC channels. VDAC channels were reconstituted into planar phospholipid membranes (diphytanoyl PC) as described in Methods. In the results illustrated, each membrane contained over 100 channels. In B, modulator-containing fraction was added to a final concentration of 20 μg/ml (crude protein) to the cis-side of the membrane. The indicated voltages refer to the cis-side. Superimposed traces of the decay in conductance following the application of voltage steps (as indicated) are shown. Note that higher voltages were applied in the control experiments to achieve similar rates of channel closure.

was converted into membrane conductance by dividing by the voltage applied. In control experiments, VDAC channels began to close at -30 to -35 mV, but was not clearly evident until -40 mV in DPPC membranes (Fig. 1). By contrast, in the presence of the VDAC modulator, channels began to close at about -15 mV and closed very well at -20 to -25 mV. High transmembrane potentials further increased the rate of channel closure and slowed down the reopening process.

The addition of the modulator-containing fraction (final protein concentration 20 μg/ml) not only greatly increased the voltage dependence of VDAC channels, but also decreased the steady-state current across the membranes. At higher protein concentrations (about

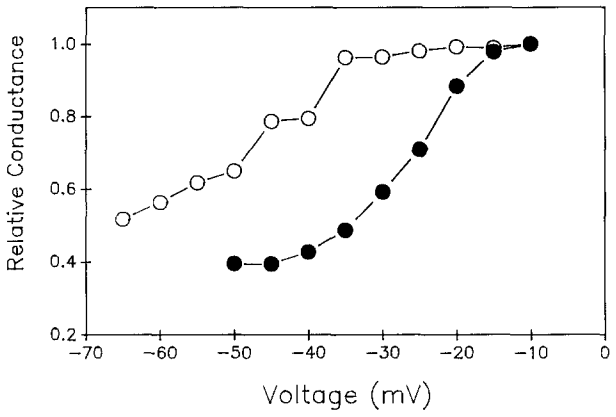

Fig. 2. The relative steady-state conductance of untreated (open circles) and modulator-treated (filled circles) VDAC channels as a function of applied voltage. The modulator-containing fraction (final concentration 20 μg/m) was added to the cis-side of the membranes. The indicated voltage refers to the cis-side. For each applied voltage, the steady-state current flowing through the channels was measured. Then the conductance was calculated from the currents and normalized to the maximum conductance (conductance at −10 mV).

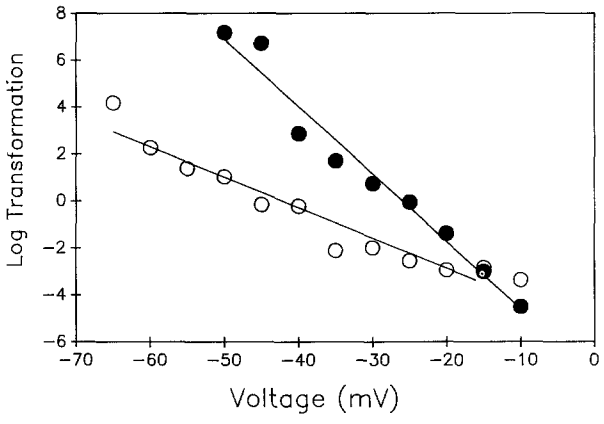

Fig. 3. Natural log transformations of the conductance voltage relationships illustrated in Fig. 2. The data were fitted to a two-state model as described in Materials Methods. The steepness of the voltage dependence (n) was obtained from the slope of each straight line. V_0, the voltage at which half of the channels are in the open state, was obtained from the intercepts on the voltage axis. For the control (open circles) n is 3.0 and V_0 is −40 mV. After addition of the modulator-containing fraction (filled circles), n increased to 7.3 and V_0 decreased to −24 mV.

100 μg/mg), some VDAC channels tended to remain in closed states and could not be reopened even when the opposite potential was applied (data not shown). As a result, a much smaller instantaneous current would be obtained, indicating that some channels remained in the closed state, or were blocked. We avoided this situation by using the results obtained with smaller amounts of modulator-containing fraction for subsequent analysis.

The increase in voltage dependence induced by the VDAC modulator is better illustrated by the conductance–voltage relationships. Figure 2 shows how the voltage dependence of the conductance of a multichannel membrane changes in the absence and the presence of the VDAC modulator. For each applied voltage, the steady-state current was measured to calculate the membrane conductance. The results were normalized by dividing the conductance values for each applied potential by the maximum conductance (all channels are open). In the control experiment, the conductance of the multichannel membrane decreased with the increasing voltage. The steep drop in conductance is around −40 mV in neutral DPPC membranes. In the presence of the modulator, a much steeper voltage dependence was observed, and a steep drop in conductance was found around −20 mV (Fig. 2).

The voltage-dependence parameters were obtained after fitting to a two-state model (Schein *et al.*, 1976; Colombini, 1989). Figure 3 shows the natural log transformation of the conductance–voltage relationship with and without the modulator. From this figure, we can see that the modulator both increased the slope of the straight line and shifted the line toward zero. This change means that the modulator increased the value of n and decreased the V_0, indicating an increase in the channel's voltage dependence. A two-to threefold increase in n (from 3.1 ± 0.3 to 7.3 ± 0.6), the measure of the voltage dependence, was observed following the addition of the modulator-containing fraction. The value of V_0, the voltage at which half of the channels are open or closed, is decreased by about twofold (from −40 ± 4 mV to −24 ± 3 mV).

For a voltage-dependent ion channel, the energy difference between the open and the closed states can be divided into two components: the voltage-dependent and the voltage-independent. The voltage-independent intrinsic conformational energy is the energy difference between the two states in the absence of an electric field. By applying the potential (V_0) at which only one-half of the channels are open, we can obtain this intrinsic energy, nFV_0. If the VDAC modulator only acted to increase the voltage dependence, it should have increased the parameter n and decreased the parameter V_0 in a compensatory way, resulting in no change nFV_0. However, the total conformational energy change also increased a little bit from

12.5 ± 1.6 kJ to 18.0 ± 1.5 kJ. This small increase may indicate that the modulator binds to VDAC channels, perhaps changing their conformation. This may account for the slow rate of channel reopening, even at zero potential.

DISCUSSION

Like other voltage-dependent ion channels, VDAC channels are known to have a positively charged sensor which can move in response to changes in transmembrane potentials. From the steepness of VDAC's voltage dependence (mammaliam VDAC), its sensor must contain at least four amino groups (Bowen *et al.*, 1985; Doring and Colombini, 1985; Adelsberger-Mangan and Colombini, 1987; Colombini, 1989). A membrane potential and perhaps the VDAC modulator can exert a force on the senor and induce a conformational change in VDAC, resulting in channel closure.

VDAC is the major permeability pathway through the outer mitochondrial membrane (Colombini, 1979; Zalman *et al.*, 1980; Freitag *et al.*, 1982; Linden *et al.*, 1982a; Mannella and Colombini, 1984). The voltage-dependent properties of VDAC channels are highly conserved in all organisms studied so far (Colombini, 1979, 1989; Smack and Colombini, 1985; Kayser *et al.*, 1989). The same is true for the functions of the VDAC modulator (Liu and Colombini, 1991). By increasing the steepness of the voltage dependence of VDAC channels, the modulating protein may act as an amplifier, controlling the metabolic flux into and out of the mitochondria and regulating the rates of cellular processes such as respiration. Channels could be opened and closed by altering the level of modulator activity. This activity change could occur as a result of protein phosphorylation, the binding or release of charge metabolites or inorganic ions, such as Ca^{2+} or Mg^{2+}, and changes in local pH.

Since VDAC channels have a large diameter and weak selectivity among small ions, it seems unlikely that a membrane potential across the outer membrane could be maintained by means of an ion pump. However, unless the charges on the macromolecules in the cytoplasm and the intermembrane space of mitochondria are equal, the small potential required to close the channels could be generated by a Donnan potential. While this Donnan potential could itself be modified under different physiological conditions, alternatively, the potential might remain constant and the activity of the modulator could be increased or decreased, resulting in VDAC opening or closure.

Some synthetic polyanions have been known to dramatically increase the voltage-dependent behavior of VDAC channels (Mangan and Colombini, 1987; Colombini *et al.*, 1987). The fact that the VDAC modulator can also increase VDAC's voltage dependence may indicate that polyanions mimic the action of this protein. The modulator may act through a negatively charged domain that interacts electrostatically with the channel's sensor or binds with part of the channel, inducing channel closure. Indeed, other major properties of the modulator's effect are identical to those of polyanions. Both act whether added to one side of the membrane or the other. If added to only one side, augmentation in voltage dependence occurs, in both cases, only when the side to which the material is added is made negative. However, there are also some differences. The VDAC modulator reduces the opening kinetics of the channel as reported for König's polyanion but unlike polyanions with no significant hydrophobic residues (e.g. dextran sulfate). The synthetic polyanions do have more potent effects on the parameters, n and V_0, but this is probably due to their higher valency. These properties are understandable on the basis of the proposed mode of action of dextran sulfate (Mangan and Colombini, 1987). The presence of the modulator, together with the possible Donnan potential, could control the metabolic flux across VDAC channels in the outer membrane and, consequently, could regulate the rate of cellular energy production.

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