Voltage-Dependent Channels Found in the Membrane Fraction of Corn Mitochondria

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ABSTRACT

Transmembrane channels have been found in the membrane fraction of corn (Zea mays W64AN) mitochondria that exhibit a remarkable resemblance to the voltage dependent anion-selective channels (VDAC) located in the outer membrane of animal (Rattus norvegicus), protist (Paramecium aurelia), and fungal (Neurospora crassa) mitochondria. These channels are distinguished from all other mitochondria in the outer membrane of corn mitochondria and by three characteristic properties: (a) the magnitude of their single channel conductance, (b) their weak anion selectivity, and (c) the nature of their voltage dependence. These findings led us to conclude that the channels present in corn mitochondria are VDAC channels. This discovery may have repercussions concerning the regulation and function of higher plant mitochondria, and the causation of higher plant excitability.

Mitochondria have an inner and an outer membrane. The inner membrane is known to be involved (among other functions) in the processes of cellular respiration, and the generation of ATP. In contrast, the exact function of the outer membrane is not as well understood. Early research on the outer membrane of isolated mitochondria indicated that the outer membrane is freely permeable to molecules as large as several thousand daltons (17, 19, 26, 27). In animals (Rattus norvegicus), protists (Paramecium aurelia), and fungi (Neurospora crassa), the permeability of the outer mitochondrial membrane has been demonstrated to be due to large transmembrane protein channels, which form 3 to 4 nm in diameter aqueous pathways through the membrane (2, 10, 12, 24). These channels are distinguished from all other channels by their location in the outer membrane of mitochondria, and by three characteristic properties: (a) the magnitude of their single channel conductance, (b) their weak anion selectivity, and (c) the nature of their voltage dependence (4, 11, 24). Channels meeting these criteria are denoted by the name VDAC2 (24).

The name VDAC describes the functional properties of the channels, and stands for voltage dependent anion-selective channel (24). These VDAC channels exist in at least two states: a high conductance or ‘open’ state, and a low conductance or ‘closed’ state (2, 4, 7, 23, 24). They are referred to as being voltage dependent because the probability of which state the channels will be in at a given instant in time is a function of the voltage difference across the membrane containing them (2, 4, 7, 23, 24). The VDAC channels are also anion selective since they preferentially allow anions to pass through them as opposed to comparably sized and charged cations (3, 24).

In higher plants, negatively stained outer mitochondrial membranes have been examined by EM. Points of stain with average center to center spacings of 4.5 nm were observed (18). Channels have been extracted from mung bean mitochondrial outer membranes whose pore size is very similar to VDAC channels (28). These findings suggest that there are large transmembrane channels in the outer membrane of higher plant mitochondria. A study of these channels could prove to be quite interesting since all mitochondria examined to date have been shown to possess VDAC channels (2, 4, 7, 23, 24). Thus, any observed differences in properties between the channels in higher plants and VDAC channels (found in the mitochondria of other life forms) might imply that higher plant mitochondria are regulated, and function in a different manner. (It has been proposed [4, 7] that VDAC may regulate mitochondrial function by controlling the flux of metabolites between the cytoplasm and the intramembrane space). If, on the other hand, VDAC’s properties were found to be conserved in higher plants, it would support the view that these properties are essential for normal mitochondrial functioning.

The finding of voltage-dependent channels in higher plants would substantiate two recent reports by Moran et al. (15, 16), which indicated that voltage-dependent channels are present in higher plants. Although voltage-dependent channels are the causal agent of excitability in animal cells, in principle, it is possible that the numerous examples of higher plant excitability (20, 22, 25) have a different molecular basis. In theory, there are many ways of generating excitable phenomena. The discovery of VDAC channels would offer further support to the hypothesis that voltage-dependent channels exist in higher plants and may be responsible for higher plant excitability. This report will show that VDAC channels do in fact exist in higher plant mitochondria, namely, corn mitochondria.

MATERIALS AND METHODS

Preparation of Mitochondrial Membrane Fraction. Intact corn mitochondria, a generous gift from Marcia Holden of the Botany Department of the University of Maryland, were separated from the roots of corn (Zea mays W64AN) seeds according to the methods of Kimber and Sze (9). The corn mitochondria were placed in a hypotonic 1 M KCl, 1 M Hepes (Na+ salt, pH 7.0) aqueous solution to promote lysing. This mixture was centrifuged in a Sorvall RC2-B equipped with an SS34 rotor at 23,600g for 30 min to obtain a corn mitochondrial membrane pellet. The supernatant resulting from this spin was carefully drawn off using a pipet. Following this, the pellet was resuspended in more lysing solution, and the previously described centrifuging procedure was repeated. The second pellet obtained was resuspended in 0.5 ml of an aqueous solution containing 1 M KCl, 1 M Hepes (Na+ salt, pH 7.0), 1% Triton X-100 (v/v), and 15% DMSO (v/v). This suspension was then stored at about 20°C. Triton X-100 was used to solubilize the protein contained within the corn mitochondrial membranes. DMSO served as a cryoprotective...
agent. The presence of DMSO and the concentration of Triton X-100 used in this study have been shown to have no effect on the properties of planar phospholipid bilayer membranes generated from asolectin (5, 6). All chemicals used were either purchased from Sigma Chemical Co., or were of reagent grade.

Formation of Bilayers and Current Measurement. The planar phospholipid bilayer membranes used in this study were generated according to the method of Montal and Mueller (14) as described in Schein et al. (24). In short, a 1% (w/v) solution of asolectin (soybean phospholipid purified according to the method of Kagawa and Racker [8]) in hexane was used to generate monolayers on the surface of the aqueous solutions in the two compartments of the chamber. These monolayers were then raised (by injecting more solution into the subphase) to construct the bilayer across a 0.15 mm diameter hole in a Saran (Dow Chemical Co.) partition separating the compartments of the chamber. Current flow (number of ions crossing the membrane per unit time) across these bilayers was recorded on a Kipp and Zonen BD41 dual pen chart recorder, and was measured with calomel electrodes under controlled voltage conditions. One aqueous compartment of the chamber (defined as the cis compartment) was maintained at 0 potential with an operational amplifier as described in Schein et al. (24). The voltage in the other aqueous compartment (defined as the trans compartment) was controlled at desired values.

Chamber Solutions. The cis and trans compartments of the chamber were filled by syringes containing aqueous solutions. The chamber was referred to as containing symmetric aqueous solutions if both compartments contained the same concentration of an aqueous solution (1 m KCl, 5 mM CaCl₂ was used in these experiments). The chamber was referred to as containing asymmetric aqueous solutions if the compartments contained two different concentrations of an aqueous solution (we used 1 m KCl, 5 mM CaCl₂ on the cis side, and 0.1 m KCl, 5 mM CaCl₂ on the trans side). The CaCl₂ was included in the aqueous solutions bathing the planar phospholipid bilayer membranes because it improves the stability of the bilayers.

Analysis of Channel Voltage Dependence. For the voltage-dependence experiments, the applied voltage was varied continuously with time using a Wavetek model 184 sweep generator. The data yielded by this technique was a plot of current as a function of applied voltage. This data was then converted to a plot of conductance (G) as a function of applied voltage (V) using a Hewlett-Packard digitizer operated by a Hewlett-Packard 85 computer. The data was digitized and the measured current was divided by the applied voltage (V) to obtain the conductance (G).

To get a more quantitative description of the voltage dependence of the channels, an analysis based on the Boltzmann distribution was performed. It was assumed in this analysis that the channels were in equilibrium at any given voltage (V), and that the channels could exist only in either an open or a closed conductance state. The results were analyzed as previously described (24) using the following equation:

$$\ln \left( \frac{G - G_{min}}{G_{max} - G} \right) = \frac{-nFV + nFV_0}{RT}$$

where G is the conductance at any voltage (V), $G_{max}$ is the maximum conductance (all channels in open conductance state), and $G_{min}$ is the minimum conductance (all channels in closed conductance state). F, R, and T are Faraday’s constant, the gas constant, and the temperature in degrees Kelvin. n is a measure of the steepness of the voltage dependence, while $V_0$ is the voltage at which half the channels are open. A plot of $\ln \left( \frac{G - G_{min}}{G_{max} - G} \right)$ versus applied voltage yields the values of n (the slope/(F/RT)) and $V_0$ (the x-intercept).

**RESULTS AND DISCUSSION**

Channel Formation. Planar phospholipid bilayer membranes were generated (as described in "Materials and Methods") in symmetric aqueous solutions. They were observed to have a very small current (number of ions crossing the membrane per unit time) of $10^{-13}$ amp-flowing across them in response to a 10 mV applied voltage. This extremely small current across the bilayers was steady, and indicated the formation of rather impermeable membranes. An aliquot of the Triton X-100 extracted corn mitochondrial membranes was added (under constant stirring) directly to the cis side aqueous solution bathing the bilayer. Usually within 20 s of this addition, we observed several uniform stepwise increases in the current flow across the bilayer, which were then followed by erratic fluctuations in the current flow (Fig. 1, A and B). The fluctuations may be due to conducting elements of an unknown nature inserting into the bilayer. The initial discontinuous increases in the current flow across the bilayer are characteristic of channel formers. Each initial current increase then represents the insertion of a transmembrane channel from the aqueous phase. The uniformity of most of the initial current increases leads one to conclude that these corn channels represent a single population.

From the initial corn channel insertions in Figure 1 (A and B), it was possible to construct a histogram of corn channel conductance by converting the current increase associated with an insertion to a conductance increase (Fig. 2). As indicated in the Figure, most of the corn channel insertions had conductance increments in the range of 3.6 to 4.2 nanoamperes (ns); 3.9 ± 0.3 nS for the major channel grouping. This range partially overlaps the range of conductance increments associated with individual VDAC channels, which is 4 to 5 nS (2, 7, 23). Corn

**FIG. 1.** (A and B), Stepwise increases in the current flow through bilayers following the addition of an aliquot of the Triton X-100 extracted corn mitochondrial membranes. Both compartments of the chamber contained aqueous solutions of 1 m KCl, 5 mM CaCl₂, while the bilayers used were generated as described in "Materials and Methods." A 10 mV applied voltage was used as the electrical driving force. The arrow (A) indicates the addition (under constant stirring) of a 2 µl aliquot of the Triton X-100 extracted corn mitochondrial membranes to the cis side aqueous solution of the chamber. This same addition was also made to the bilayer (B). Current flow through the bilayers was essentially 0 prior to the stepwise increases.
channel conductance increments smaller and larger than the approximate average were also observed. The smaller conductance increments are also seen in VDAC channel conductance histograms, and may represent individual corn channels inserting in a lower conductance state as postulated for VDAC channels (2). The larger conductance increments might be due to the fact that the naturally occurring membrane arrangement of these channels is in triplet units as was found to be the case with Neurospora crassa VDAC channels (13). A possible explanation for the rarity of these large insertions might be that the Triton X-100 solubilized the triplets into doublets and singlets.

**Channel Voltage Dependence.** Following the insertion of corn channels into the bilayer as shown in Figure 1 (A and B), it was observed that the channels shifted from a high conductance or open state to a low conductance or closed state when 35 mV was applied (inset A, Fig. 3). Note that when the 35 mV was applied, the current flow across the bilayer first increased instantaneously due to the increased electrical driving force, and then decreased stepwise as the individual corn channels shifted to the lower conductance state. Due to the stochastic nature of the channels, the rates of channel 'opening' and 'closure' were not well defined if the membrane contained only a few channels. The presence of many channels in the bilayer alleviated this problem. With a large number of corn channels present in the bilayer, the shift from the open to the closed state when 30 mV was applied was seen as a smooth decrease in the current flow across the bilayer (Fig. 3). Observe that when the applied voltage was reduced from 30 mV to 10 mV, the current level returned to its value prior to the 30 mV pulse showing that the corn channels shifted from the closed to the open state at a rate much faster than the recorder's response time (inset B, Fig. 3). Thus, the corn channels opened from the closed state more slowly than from the close to the closed state, which is also the case with VDAC channels (4).

With a large number of corn channels in the bilayer, the applied voltage was varied from 0 mV to + and − 55 mV at a rate of 12.2 mV/min using the sweep generator. This procedure yielded a plot of current flow across the bilayer versus applied voltage. The current flow was then converted to conductance (as described in "Materials and Methods") to make the plot shown in Figure 4 of conductance (G) versus applied voltage (V). As the applied voltage was increased from 0 mV to 55 mV in either the positive or negative direction, the conductance of the corn channels decreased (Fig. 4). This means the corn channels are voltage dependent since the probability of which state (open or closed) the channels will be in at a given instant in time is a function of the voltage difference across the membrane containing them. VDAC channels are also voltage dependent in this fashion (4). The steepness of the corn channel voltage dependence (n) was found to be 3.8 ± 0.1 (eight estimates), while the applied voltage at which half the channels were open (V1/2) was 20 ± 2 mV (eight estimates) (see "Materials and Methods"). The previously reported values for VDAC channels (measured under the same conditions) ranged from 3.5 to 4.5 for n, and 20 to 25 mV for V1/2 (6, 24).

**Channel Selectivity.** The selectivity of the corn channels was determined with asymmetric aqueous solutions (see "Materials and Methods") in the compartments of the chamber. The corn channels were inserted into the bilayer directly from the aqueous phase as before except with no applied voltage. A current flow through the bilayer was observed as the corn channels inserted in a stepwise fashion even though there was no applied electrical driving force. A +10.2 mV (positive on the high salt side) applied voltage was required to bring the current flow through the bilayer to 0 (mean of two estimates, 10.0 and 10.4). This voltage is known as the reversal potential, and can be used to supply information on channel selectivity. Using the Goldman, Hodgkin, and Katz equation and the corn channel reversal potential, the ratio of chloride ion to potassium ion permeability was determined to be 1.7 (the concentrations in the Goldman, Hodgkin, and Katz equation were converted to activities using activity coefficients from Robinson and Stokes [21]). Thus, the corn channels are weakly anion selective just like VDAC channels whose ratio of chloride ion to potassium ion permeability is also 1.7 (6).
For of two conductance.

This data obtained in the form of a plot of current flow across the bilayer versus applied voltage. Each side (positive and negative) of the plot represents the numerical average of four separate conductance versus applied voltage plots obtained from four different experiments. Originally, the data yielded by these experiments was in the form of a plot of current flow across the bilayer versus applied voltage. This data was converted as described in "Materials and Methods" to a plot of conductance versus applied voltage. For each set of points, the conductance was normalized to the highest observed conductance (96 nanosiemens (nS) for the negative side, 76 nS for the positive side). The two highest observed conductances were not equal in magnitude since the bilayers analyzed contained different numbers of channel layers. The sign of the applied voltage refers to this compartment of the chamber. For these experiments, both compartments of the chamber contained aqueous solutions of 1 m KCl, 5 mm CaCl₂, while the bilayers used were generated as described in "Materials and Methods."

**Significance of Findings for Higher Plants.** It was demonstrated that the corn channels possess functional properties (voltage dependence and anion selectivity) identical to those displayed by VDAC channels. Individual VDAC channel conductance increments were also shown to be essentially equal in magnitude to individual VDAC channel conductance increments. These findings make it possible for us to conclude that the corn channels are VDAC. The presence of VDAC channels in corn mitochondria indicates that if VDAC regulates mitochondrial function in other life forms, similar regulation may occur in higher plants. The remarkable similarity between corn VDAC channels and those previously studied indicates a very tight conservation of function as these organisms evolved from a common ancestor. The alternative possibility of convergent evolution cannot be ruled out, but seems unlikely in view of the apparent ubiquitous nature of VDAC channels (there is no example of mitochondria lacking this channel [2, 4, 7, 23, 24]). Indeed, there exists a family of channels, called porins, found in the outer membranes of gram negative bacteria (such as Escherichia coli), which possess some resemblance to VDAC (1, 3). If one adheres to the endosymbiotic hypothesis of mitochondrial origin, porins may resemble the ancestral channel from which VDAC evolved. This conservation of VDAC's characteristics underscores its biological importance.

The excitability found in higher plants closely resembles the excitability seen in animals. For instance, action potentials strikingly comparable to those found in animals have been recorded in the petiole of Mimosa pudica leaves (22), and on the inner surface of Dionaea muscipula trap lobes (25). In animals, voltage-dependent channels are the causal agents of excitability. In higher plants the causal agents can only be surmised. The existence of corn VDAC channels confirms very recent reports (15, 16) of voltage-dependent channels in higher plants. Thus, perhaps, voltage-dependent channels may underlie the excitability observed in higher plants.

In summary, VDAC channels have been found in the membrane fraction of corn mitochondria that are remarkably similar to the VDAC channels located in the outer membrane of animal, protist, and fungal mitochondria. This finding indicates further areas of functional similarity between the physiology of higher plants and the other life forms.

**Acknowledgment—** We would like to express our gratitude to Marcia Holden of the Botany Department of the University of Maryland whose generous gift of intact corn mitochondria made this work possible.

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