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Mitochondrial Channel Activity Studied by Patch-Clamping Mitoplasts

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Abstract

Patch-clamping mitoplasts, we have observed a complex pattern of conductance transitions. This report discusses primarily the 45, 120–150, 350, and 1,000 pS transitions.

Key Words: Mitochondrial channels; inner mitochondrial membrane; channels.

Introduction

Patch clamp studies of the inner mitochondrial membrane can potentially provide a wealth of information on ion transport, proton pumping and their control, as well as other membrane events relevant to the energy transduction of oxidative phosphorylation.

Sorgato *et al.* (1987) were the first to report patch-clamping of the inner mitochondrial membrane and describe a single, slightly anion-selective high-conductance channel of 107 pS in 150 mM KCl. Furthermore, they report a 350 pS channel attributed to the outer mitochondrial membrane.

We have examined the properties of excised and attached patches from mitoplasts (i.e., mitochondria which have been treated to remove the outer membrane) prepared from mouse liver mitochondria and suspended in 150 mM KCl media. This presentation will report on conductance levels observed in these experiments.

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Methods

Preparations

Our patch-clamp studies were conducted with mitoplasts prepared from isolated mouse liver mitochondria. Initially, the mitochondria were isolated from the liver of young mice maintained on a diet containing cuprizone (Bowman and Tedeschi, 1983). Since mitochondrial suspensions from normal young mouse liver contain some relatively large mitochondria (some are 2–4 μm in diameter) we used untreated mice in all later experiments. The results obtained with the two different mouse preparations were indistinguishable. After isolation, in a few experiments we used the French pressure cell method of Decker and Greenawalt (1977) to remove the outer membrane from either normal mouse or rat liver mitochondria. This preparation has been well characterized biochemically and with electron microscopy. For the results presented in this paper, mitoplasts were prepared by the simpler method of Gupte *et al.* (1984). Both mitoplast preparatory techniques provided similar results with patch-clamping. With the osmotic procedure, the mitochondria were first isolated (Bowman and Tedeschi, 1983) and suspended in 0.3 osmolal sucrose, 5 mM HEPES, pH 7.4. After a dilution of 1 : 20 with 2 mM HEPES, pH 7.4, they were centrifuged at 650 × *g* for 5 min and resuspended in our experimental medium, 150 mM KCl, 5 mM HEPES, pH 7.4.

Electronics and Recording

The electronics used in patch-clamping have been described previously (Tedeschi *et al.*, 1987). Generally, data were displayed during the experiments on a Hitachi digital storage oscilloscope (model VC 6020, Hitachi Denshi Ltd., Woodbury, New York). They were recorded on video tape using a digitizer (VR10 digital data recorder, Instrutech Corp., Mineola, New York) and an Emerson VCS 966A video cassette recorder. A filtering device (model 902, Frequency Devices, Inc., Haverville, Massachusetts) was used to filter in the range of 1–4 kHz. The results were displayed and analyzed with an IBM XT or an IBM AT-compatible computer generally using IPROC software (courtesy of C. Lingle, Washington University) and Strathclyde Electrophysiological Data Analysis software (courtesy of J. Dempster, University of Strathclyde, England).

Patch-Clamping

The patch-pipettes had openings of 0.2–0.5 mμ in diameter and resistances generally of 10–40 MΩ. Mitoplasts prepared from normal mitochondria were

2–5 μm in diameter and the patches ranged from 1 to 20 GΩ. Typically the data shown in this paper came from the most stable patches, with resistances of 2 to 7 GΩ. Excised patches were inside-out (i.e., matrix side in the external medium) since they generally showed the same I–V curve asymmetry as the mitoplast-attached patches. All voltages reported are in relation to the interior of the mitoplast. Conductance levels were calculated from the slope of amplitude of current transitions vs. voltage plots.

Results

Upon patch-clamping osmotically prepared mitoplasts, the current–voltage dependence (i.e., the I–V curve) typically changed with time from a low activity patch with a symmetrical response to voltage (shown in Fig. 1A) to one with more activity. A delay in the activation of channels during patch-clamping has been observed before, for example in high-conductance anion-selective channels of Schwann cells (Gray *et al.*, 1984), and has been ascribed to the loss of endogenous modulator. Frequently an asymmetry in the I–V curves also developed with time, as shown in Fig. 1B. This asymmetry indicates the presence of voltage-sensitive channels, usually showing an opening in the negative range of potentials and a closing in the positive range. The observed variability of the I–V curves may be the result of the action of regulatory factors (see Discussion).

Representative current traces from patch-clamped mitoplasts are shown in Fig. 2A–E. A variety of conductance levels are observed, approximately 10–20, 45, 80, 120–150, 200, 350, and 1000 pS. Infrequent transitions corresponding to 400–900 pS (not shown) have also been recorded. Several conductance levels are distinguishable within the same patch as current transitions of different amplitudes at a constant voltage (see Fig. 2A, lines 1, 5, 6). The complex pattern of channel activity occurs in bursts and is followed by quiescent periods (see Fig. 2A, line 4). At times a single conductance level is observed for some time (Fig. 2A, lines 2 and 3), and these sectors were analyzed in more detail. Approximately the same pattern was observed in excised and mitoplast-attached patches. In Fig. 2A, as in most patches, the most commonly observed current transition corresponds to the 45 pS level. A 10–20 pS transition appears on top of the open 45 pS level (beginning of line 1, Fig. 2A). Larger transitions of 80–150 pS are shown in lines 5 and 6 of Fig. 2A.

A survey of the ion selectivity and voltage dependence of the conductance transitions is in progress. Ion selectivity was assessed from the reversal potential after imposing a K^+ and/or Cl^- gradient across excised patches (see Hille, 1984). A qualitative summary of our results is shown for the

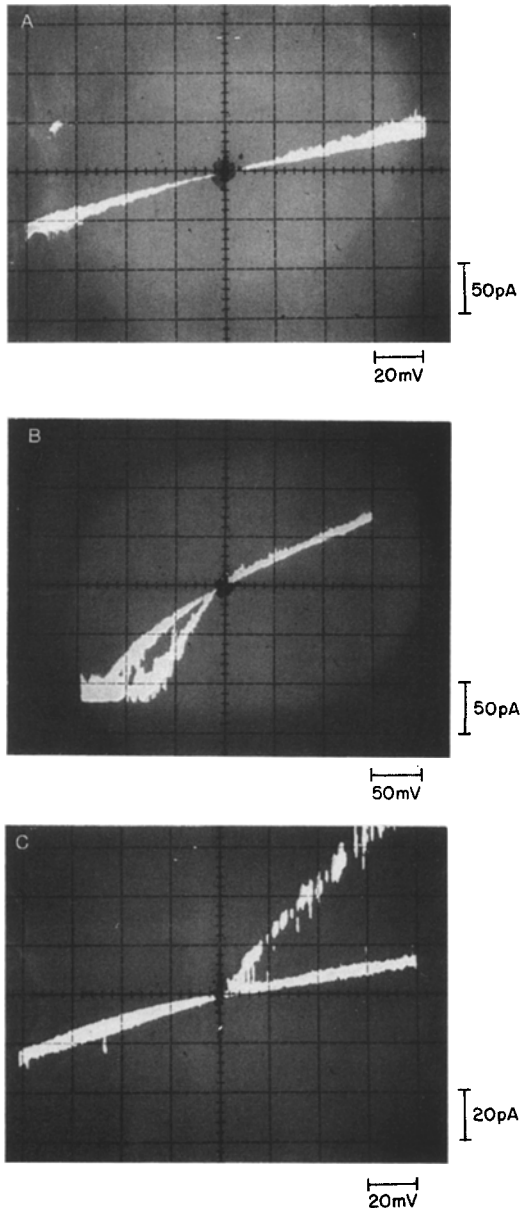

Fig. 1. Current–voltage curves. The origin of the axes indicates zero current and voltage. Current was filtered at 1 KHz. (A) Symmetrical *I–V* curve of an attached mitoplast patch. (B) Asymmetrical *I–V* curve of an excised patch in symmetrical KCl media. (C) Single-channel *I–V* curve of the ~ 1000 pS level from an excised patch in symmetrical KCl media. The upper line in the positive-voltage region reflects the open-channel current level.

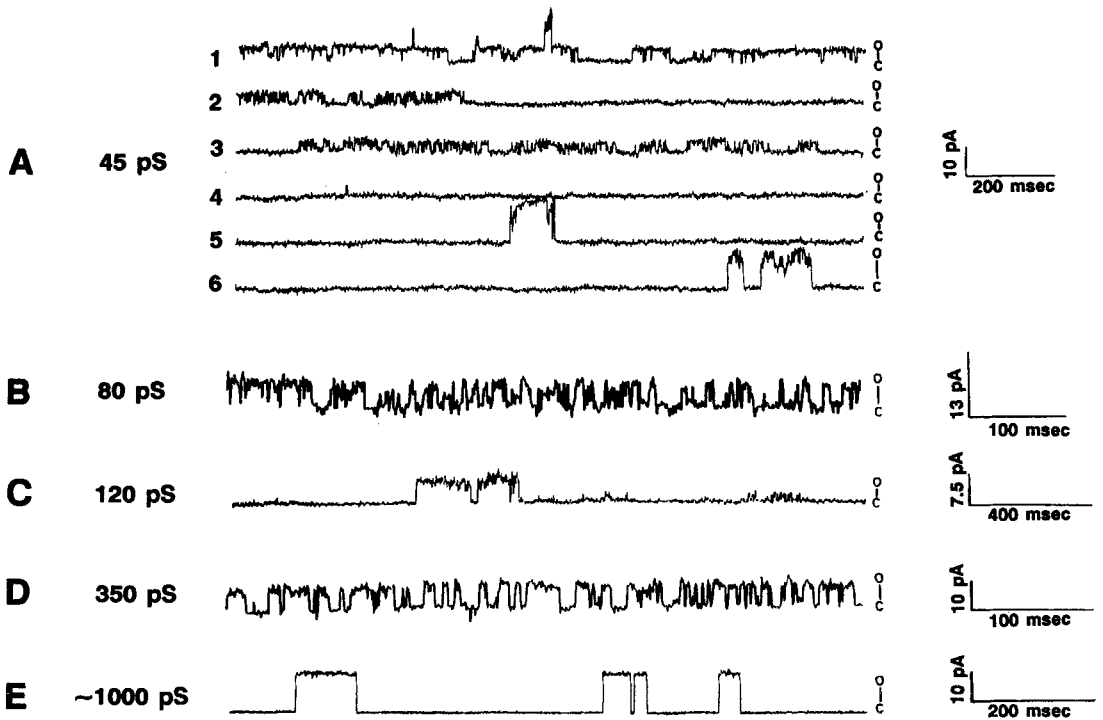

Fig. 2. Representative current traces. O indicates open-channel and C indicates closed-channel current levels. Filtration was 1 KHz unless otherwise noted. All excised patches were in symmetrical KCl media. (A) Continuous current trace at +75 mV from attached mitoplast patch showing transitions to several levels at 2 KHz filtration. Activity, predominantly of the 45 pS level (~3 pA transitions in lines 1–3), occurs in bursts followed by quiescent periods (line 4). The 10–20 pS level is seen at the beginning of line 1 on top of the open 45 pS level. Transitions of 80 to 150 pS are seen in lines 5 and 6. (B) Current trace at +75 mV corresponding to the 80 pS level from an excised patch. (C) Current trace at +40 mV showing transitions corresponding to the 120–150 pS level from an excised patch. (D) Current trace at +20 mV showing transitions corresponding to 350 pS activity from an excised patch. (E) Current trace at +10 mV showing transitions corresponding to the approximately 1,000 pS level in an excised patch.

Table I. Summary for Four Conductance Levels[a]

Conductance in 150 mM KCl	Selectivity	Voltage dependence
45	Slightly anionic **	None
120–150	Slightly cationic **	* (open −, close +)
350	Slightly cationic *	** (open −, close +)
approx. 1000	Not done	*** (open +, close −)

[a]Asterisks indicate relative selectivity or voltage dependence, plus or minus indicate polarity of voltage.

45, 120–150, 350, and approximately 1000 pS levels in Table I. The lowest conductance level (10–20 pS) is often obscured by the activity of other channels, and we have as yet been unable to study it in detail. For the same reason, levels below 100 pS are difficult to examine in the presence of activity from higher conductance levels (e.g., 350 pS). The ~1000 pS level has not been examined for ion selectivity since it is recorded infrequently. The voltage dependence of the ~1000 pS level is shown as a single-channel *I–V* curve in Fig. 1C. It is closed with negative voltages but shows frequent openings (upper trace) with positive voltages. The 45 pS level, the most frequently observed level at all voltages, does not appear to be voltage dependent under the conditions of our experiments. It is slightly anion selective. The 120–150 pS and the 350 pS levels are slightly cation selective and show a variable voltage dependence. When asymmetric *I–V* curves (e.g., Fig. 1B) are obtained, the 120–150 and/or the 350 pS levels close with positive voltage steps and open with negative voltage steps. In general, ion selectivity of the conductance levels is weak, the relatively permeability ratios of K^+/Cl^- or Cl^-/K^+ occurring in the range of 3–10.

Discussion

We attribute the channel activity detected by patch-clamping mitoplasts to the inner mitochondrial membrane for the following reasons. (a) The extensive swelling used to prepare mitoplasts (Gupte *et al.*, 1984) in most experiments precludes the presence of large intact portions of outer membrane. (b) Mitoplasts prepared with the French press method have virtually no outer membranes (Decker and Greenawalt, 1977). Results obtained patch-clamping these mitoplasts (Kinally *et al.*, unpublished results) are similar to those obtained with the osmotic swelling method. (c) Our patch-clamp studies of outer mitochondrial membranes, carried out with intact mitochondria and isolated outer membranes (Tedeschi *et al.*, 1987; Kinnally *et al.*, 1987, 1989b), indicate that these membranes have much lower resistances than those

observed with mitoplasts. Measured outer membrane resistances in the range 10–500 MΩ (in a medium containing 10 mM KCl) are consistent with the high concentration of voltage-dependent anion-selective channels (VDAC or mitochondrial porin) expected from the concentration of its constituent protein (Freitag *et al.*, 1982; De Pinto *et al.*, 1987). (d) The pattern of mitoplast conductance levels is affected by metabolism. For example, the addition of succinate to rotenone-inhibited mitoplasts often produces an increase in channel activity (Kinnally *et al.*, 1989a). Although none of these criteria are conclusive, taken together they argue strongly that we are patch-clamping the inner mitochondrial membrane.

There is, however, considerable uncertainty about the original location of the observed channels. The methods of preparation used in this study do not completely exclude the presence of pieces of outer membrane attached to the mitoplasts. Therefore, the transfer of channel proteins from outer to inner membrane is conceivable (for example, by fusion of the two membranes during micromanipulation). Similarly there is evidence for specialized contact sites containing channels between the inner and outer membranes (Saito *et al.*, 1974; Brdiczka *et al.*, 1986; Ohlendieck *et al.*, 1986; Kottke *et al.*, 1988), which change dynamically with physiological conditions (Knoll *et al.*, 1983). Furthermore, the inner mitochondrial membrane itself may be functionally heterogeneous (Brdiczka and Reith, 1985). Specialized structures resembling gap junctions have been identified at contact sites between mitochondria (Harris, 1979; Bakeeva *et al.*, 1977, 1983, 1985; Amchenkova *et al.*, 1988). In addition, similar channel-like projections have been observed in mitochondrial outer membranes (Mannella *et al.*, 1984; Ribeiro, 1988). The demonstration *in situ* of communication between mitochondria in clusters (Amchenkova *et al.*, 1988) speaks for a connection between the matrix spaces of adjoining mitochondria and hence the probable presence of intermembrane channels. The gap-junction-like regions as well as the contact sites between inner and outer membranes may be present in our mitoplast patches.

Since occasionally most or all the conductance levels appear in the same patch (e.g., Fig. 2A), it is highly unlikely that they represent activities of different classes of mitochondria (Bygrave *et al.*, 1978).

Our results differ significantly from those of Sorgato *et al.* (1987) who report the presence of two channels, of 107 and 350 pS, in preparations of liver mitochondria from cuprizone-treated mice. More recently they confirmed the presence of the 107 pS channel in mitoplast preparations from normal mice and in reconstituted liposomes and black lipid membranes (Sorgato *et al.*, 1989). Evidence for the 107 pS channel was obtained by patch-clamping mitoplasts prepared by an osmotic method which differs significantly from ours. The 350 pS channel was detected by using intact

mitochondria, and the result was attributed to patch-clamping the outer membrane. We observed multiple conductance levels (10–1000 pS) using mitoplasts. The differences in the preparative procedures presumably may account for the activation of different channels. As noted above, the channel activity increases with time, which may be the result of the loss of regulatory factors, as suggested by Gray *et al.* (1984) for patches excised from the plasma membrane of Schwann cells. It is conceivable that different factors are associated with different channel activities. Holden and Colombini (1988) have isolated an endogenous factor which modifies profoundly the activity of VDAC incorporated in bilayers. Perhaps similar regulators play a role in the inner membrane.

Are the observed multiple conductance levels separate channels or do they reflect subconductance levels of one or more channels? Alternatively, are they cooperative aggregates of identical channels that are functioning in parallel? We had hoped to distinguish between these possibilities through characterization of ion selectivity and voltage dependence of the conductance levels. However, this approach may not be feasible with mitochondria. Most channels maintain the same ion selectivity in different subconductance levels (e.g., Hamill and Sakmann, 1981; Labarca and Miller, 1981). Channels responding in parallel generally exhibit the same voltage dependence and ion selectivity (e.g., Geletyuk and Kazachenko, 1985). In contrast, the mitochondrial outer membrane channel VDAC changes from anion- to cation-selective when it switches from open to partially closed subconductance states (Colombini, 1980). In addition, Thieffry *et al.* (1988) have described a mitochondrial channel whose conductance levels have different voltage dependences.

The possible physiological role of inner mitochondrial membrane channels is intriguing. Sorgato *et al.* (1989) have addressed the possible involvement of the F_0 portion of the ATP-synthase. In addition, as mentioned above, there is evidence of channels connecting contiguous mitochondria, permitting coordinate function and regulation of multimitochondria units which can extend in the cytoplasm for several micrometers (e.g., Amchenkova *et al.*, 1988). The presence of channels is suspected in the junctions between inner and outer membrane. These could serve as avenues for the passage of solutes from the cytoplasm to the mitochondrial matrix. Singer *et al.* (1987) have suggested that protein transport through membranes requires the presence of water-filled channels, and the junction sites could provide such channels. Since the proteins are likely to be delivered to specific mitochondrial domains, more than one protein transport channel may be present. There is considerable evidence from other kinds of experiments for the presence of various channels in the mitochondrial inner membrane. The uncoupling protein of brown adipose tissue mitochondria (Nicholls and

Locke, 1984; Himms-Hagen, 1985) implicated in thermogenesis is thought to function as a proton channel in short-circuiting gradients produced by metabolically dependent H^+ efflux. However, a general role of this protein in other tissues is unlikely since it has been shown by immunological procedures to be unique to brown fat (Cannon *et al.*, 1982; Ricquier *et al.*, 1983). There is, however, considerable evidence for inner membrane channels involved in the transport of anions (e.g., Crompton, 1985; Riley and Pfeiffer, 1985; Garlid and Beavis, 1986). Stretch-activated channels have been demonstrated in a variety of systems, including microorganisms (Sachs, 1986; Saimi *et al.*, 1988), and they may be activated by the motion of elements of the cytoskeleton or alternatively by osmotic events. These possibilities should also be considered in mitochondria. The demonstration of interactions between cytoskeletal elements and mitochondria (e.g., see Lindén *et al.*, 1989) is particularly interesting and may suggest a number of possible physiological interactions. However, so far we have been unable to demonstrate stretch-activated conductance dependence in our preparations.

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