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Research Paper

Voltage Activation of Heart Inner Mitochondrial Membrane Channels

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The patch clamp records obtained from mitoplast membranes prepared in the presence of a calcium chelator generally lack channel activity. However, multiconductance channel (MCC) activity can be induced by membrane potentials above ±60 mV [Kinnally *et al.*, *Biochem. Biophys. Res. Commun.* **176**, 1183–1188 (1991)]. Once activated, the MCC activity persists at all voltages. The present report characterizes the activation by voltage of multiconductance channels of rat heart inner mitochondrial membranes using patch-clamping. In some membrane patches, the size of single current transitions progressively increases with time upon application of voltage. The inhibitor cyclosporin has also been found to decrease channel conductance in steps. The results suggest that voltage-induced effects which are inhibited by cyclosporin Aare likely to involve either an increase in effective pore diameter or the assembly of low-conductance units. In activated patches, we have found at high membrane potentials (e.g., 130 mV) changes in conductance as high as 5 nS occurring in large steps (up to 2.7 nS). These were generally preceded by a smaller transition. Similar results were obtained less frequently at lower voltages. These results can be explained on the assumption that once assembled the channels may act in unison.

KEY WORDS: Inner mitochondrial membrane; channels; voltage activation; assembly; cyclosporin; patch-clamp; permeability transition pore.

INTRODUCTION

Evidence for high-conductance channel activity with multiple substates (MCC) in mouse liver mitochondria has been obtained using patch-clamp techniques (Kinnally *et al.*, 1989; Petronilli *et al.*, 1989, for review see Kinnally *et al.*, 1992). It has been suggested that MCC activity is responsible for the Ca^{2+}-activated permeability transition observed in mitochondrial suspensions (Kinnally *et al.*, 1991; Szabó and Zoratti, 1991, 1992). In our hands, MCC activity was observed if the mitoplast preparation was done in the presence of (endogenous) Ca^{2+} (Kinnally *et al.*, 1991). Alternatively, if mitoplasts were prepared in the presence of a calcium chelator and MCC was electrically silent, it could be activated by applying a membrane potential of sufficient magnitude. Once activated, the MCC activity was recorded at all voltages with transitions ranging from 40 to as much as 2,600 pS (Zorov *et al.*, 1991) in rat heart inner mitochondrial membranes. One of the possible explanations for the activation process is that the higher conductance levels result from the assembly of lower conductance channels. Previously, we have reported with activated MCC (Zorov *et al.*, 1991): (a) negative voltage steps induced occupancy of progressively higher conductance levels, whereas positive voltage steps induced occupancy of progressively lower conductance levels, and (b) the introduction of the inhibitor amiodarone induced occupancy of corresponding lower conductance levels. The present study confirms

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some of the characteristics of high-conductance transitions of activated MCC and examines the initial events of voltage activation of MCC in membrane patches initially lacking channel activity (Kinnally *et al.*, 1991).

MATERIALS AND METHODS

Preparation of the Mitoplasts

Hearts from male rats of the Sprague–Dawley strain (100–250 g) were homogenized using a glass and Teflon homogenizer of the Potter–Elvehjem type in a medium containing 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, and 1 mM EGTA, pH 7.4. Large mitochondria were isolated as previously described (Bowman and Tedeschi, 1983), and mitoplasts were prepared from these mitochondria using the French-press method of Decker and Greenawalt (1977). After sedimentation of large mitochondria, the pellet was resuspended in 15 ml of 460 mM mannitol, 140 mM sucrose, and 10 mM HEPES, pH 7.4, kept on ice for 10–15 min and then subjected to 2,000 psi using the French press to remove the outer membrane. The mitoplasts were diluted by an equal volume of 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, and 1 mM EGTA, pH 7.4, and kept on ice for 5–10 min. They were centrifuged at 10,000 *g* for 5 min and resuspended in 3 ml of 150 mM KCl and 5 mM HEPES, pH 7.4. The procedures used (see Kinnally *et al.*, 1991) produced preparations yielding current records with no significant channel activity when clamped at or below ± 60 mV. We refer to these as "silent membrane patches."

Patch Clamping

About 10 μl of mitoplast suspension was placed on a glass slide. After several minutes the mitoplasts attached to the slide were washed with the patching medium containing 150 mM KCl, 5 mM HEPES, 1 mM EGTA, and 0.95 mM $CaCl_2$ (about 6×10^{-7} M free Ca^{2+}), pH 7.4, at room temperature (approximately 25°C). In this study only excised mitoplast membrane patches were studied. These were presumed to be in the inside-out configuration, since channel activity had the same voltage dependence in attached or excised patches (see Kinnally *et al.*, 1989). The pipettes contained the same medium and their resistance was 20 to 40 MΩ. The reference electrode consisted of a Ag–AgCl wire connected to the bath through a bridge containing the medium in 2% agar. Patch pipettes were formed from 1.0 mm diameter capillary Pyrex tubes (Corning 7740 glass, World Precision Instruments, Inc. New Haven, Connecticut 06513) using a horizontal puller (Sutter Instruments Co. Model PC-84).

A Dagan 3900A patch clamp amplifier in the inside-out mode was used under voltage clamp conditions. The current (bandwidth of 10 kHz) and voltage outputs were digitized with an Instrutech VR-10 digital data recorder (Mineola, New York) and recorded on video tape. Subsequent computer analysis of stored current signals was done at a bandwidth of 2–4 kHz obtained with a low-pass filtering device (Frequency Devices, Haverhill, Massachusetts 01830, model 902) with a sampling frequency at least twice that of the cut-off filter. The computer analysis of the data used Strathclyde Electrophysiological Data Analysis software (courtesy of J. Dempster, University of Strathclyde, UK) with a 2801A D/A board from Data Translation (Marlboro, Massachusetts). Typically, cyclosporin was delivered in a solution of the usual medium, by perfusion of 3 ml through a 0.5 ml bath. Controls were carried out by the same procedure but without the drug and no effect was observed. The quantity nP_O was calculated from amplitude histograms from the ratio of percent time at open current levels over the total time. In this paper, upward deflections are openings with positive voltages and downward deflections are openings with negative voltages.

RESULTS

MCC (multiple conductance channel) activity is characterized by the observation of multiple conductance levels and a nS peak conductance. The presence of MCC activity requires either isolation in the absence of a calcium chelator or activation generally at voltages higher than ± 60 mV. The activity may involve more than one channel, and the transitions range from 40 to 2,600 pS. While we observe transitions above 500 pS in over 50% of heart mitoplast patches (n = 25 randomly selected), transitions above 1 nS are seen in about 20% of the membrane patches. More infrequently, we have seen single transitions as high as 5 nS. Besides the inactive, electrically silent form of MCC, we have observed three distinct patterns of activity: (a) conventional opening and closing at various levels; we have recognized a minimum of nine

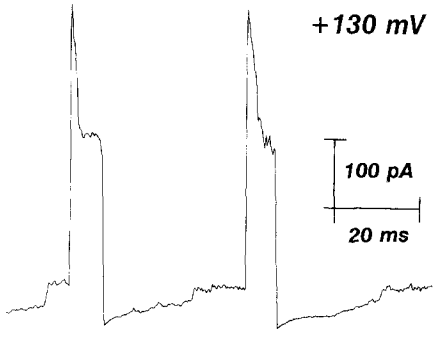

Fig. 1. Current trace of high conductance channel at a bandwidth of 4 kHz with 8 kHz sampling. The record represents two transitions selected at random from 10 similar records within a 750 ms period.

levels; (b) opening and closing in large steps of 200–600 pS, in response to voltage, where negative voltages favor occupancy of higher conductance levels, whereas positive voltages generally favor lower conductance levels; and (c) maintenance of the closed or open state of 1–1.5 nS for long periods of time (seconds or minutes) after reaching that state either by single transitions or multiple steps.

We have suggested that the opening and closing in steps corresponds to an assembly process and the opening in large steps corresponds to the various channels acting in concert possibly by a cooperative effect (Zorov *et al.*, 1991). The latter observation could be explained equally well by assuming an increase in the effective pore diameter. The upper limit in the size of the transition is of interest in establishing the mechanism of MCC activity (e.g., the minimal number of aggregating subunits). Similarly the voltage-induced transitions during the activation process may help elucidate the organization of MCC. The next two sections describe experiments examining these two questions.

Large Transitions

After activation of MCC (usually with potentials at or above ± 60 mV or calcium during preparation) we have observed in the activated patches multi-step transitions such as that shown in Fig. 1. We observe similar patterns less frequently at much lower voltages ($\pm$ 10–80 mV). In Fig. 1 the peak conductance was approximately 3.2 nS at 130 mV. A smaller step preceded a 2.8 nS transition. The closing also occurred in two steps, a smaller step followed by a single transition of approximately 1.8 nS. Openings and closings such as these occur repeatedly (e.g., in this region of the current trace we observed 10 approximately equivalent consecutive transitions in 750 ms).

Stepwise Activation and Inactivation

In order to study voltage activation, membrane potentials were applied to silent membrane patches (see Materials and Methods) as shown in Fig. 2. The size of the transitions shown in Fig. 2A increased with time at constant voltage as summarized in Fig. 2B. The size generally reached a limiting steady-state value of approximately 1 nS. Results similar to those of Fig. 2 were obtained at higher voltages as shown in Fig. 3. Typically the increase in conductance occurred much faster at higher voltages. The time course of the transition was fitted by a trial and error procedure to a single exponential ($Y = a + be^{cx}$, where Y is transition size, pA; x is time, s; a and b are empirical constants, and c is the time constant). We did not attempt to examine equations that would account for the steady state peak conductance. We have found that the time constant c increases from 0.05 to 2.57 as the absolute magnitude of the membrane potential was increased (V = 40, 60, 90, 170 mV) in different patches with similar peak conductance (1 nS). There was also an effect on the slope b which decreased from 12.0 to 0.08 in the same experiments as the voltage increased.

Cyclosporin has been found to inhibit multi-conductance channels (Szabó and Zoratti, 1991). The inhibition of large transitions and occupation of lower levels proceeds in steps as illustrated by the current traces of Fig. 4A. The decrease in the probability of opening (nP_O) to higher conductance levels is shown in Fig. 4B. The occupancy of high conductance levels (e.g., 200 and $\geqslant 300$ pS), predominant before cyclosporin addition, is replaced by occupancy of the lower levels. The conductance level steps seen during the time course of cyclosporin inhibition correspond to those observed with voltage activation in other experiments (e.g., compare Fig. 2A and 4A). Companion experiments in which cyclosporin was not present in the perfusion medium did not show this inhibition.

DISCUSSION

We have presented data showing a voltage activation of nS transitions as a progressive increase in transition size with time after the application of a voltage step to predominantly silent patches. Typically, the maximum transition size was about 1 nS

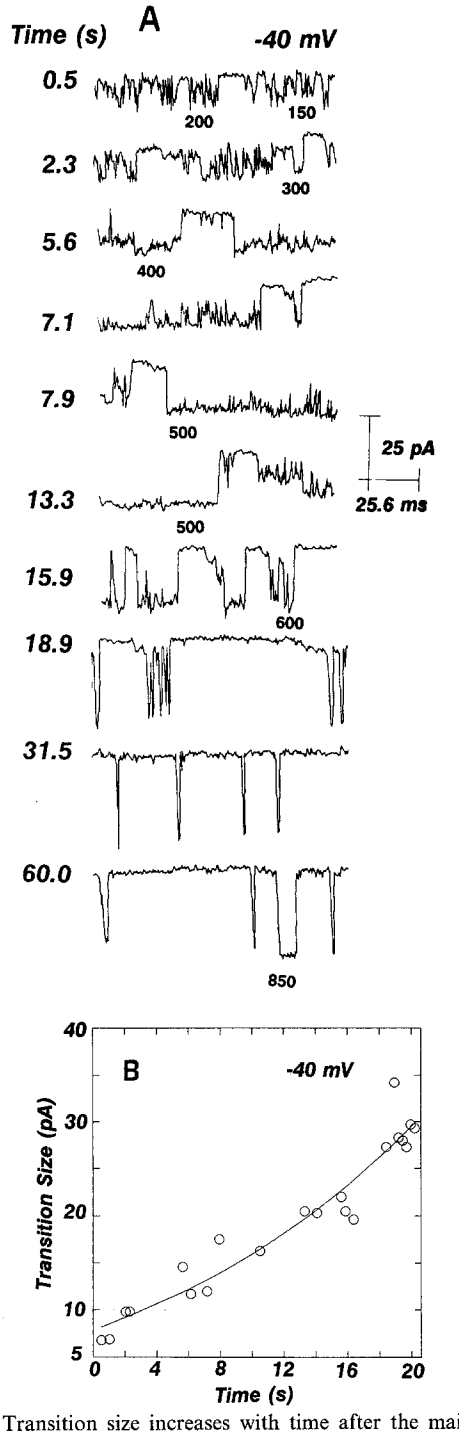

Fig. 2. Transition size increases with time after the maintained application of −40 mV. A. Current records at times after the potential is applied ($t = 0$) as indicated at 2 kHz bandwidth and 4 kHz sampling. B. Transition size (current, pA) as a function of time. Each point indicates a current level averaged from single amplitude transitions. Since a short time span had to be used, these did not differ significantly from the amplitude histograms. The line represents the best fit for the equation $Y = a + be^{cx}$, where Y is the current, a and b are empirical constants, and c is the time constant, and x is time. In this figure $a = 4.13$, $b = 12.0$, and $c = 0.05\,s^{-1}$. The peak transition size at steady state (38 ms) was approximately 34 pA.

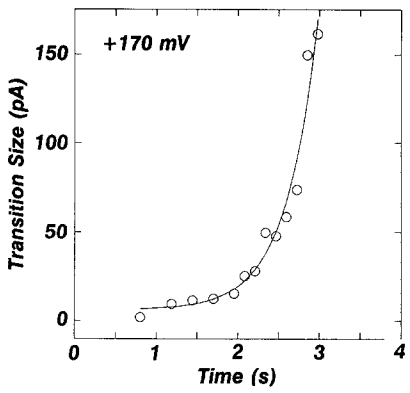

Fig. 3. Best fit of current vs. time for an experiment equivalent to that of Fig. 2 but at +170 mV. The constants are $a = 6.12$, $b = 0.08$, and $c = 2.57\,s^{-1}$. Steady state reached in 3 s showed a peak transitions size of ~170 pA (1 nS).

but those up to about 3 nS were observed. The data can be fitted at least approximately with an exponential equation in which the time constant increased with increased voltage. Inhibition of MCC activity by cyclosporin typically results in a stepwise decrease in predominant conductance level and elimination of large transitions, e.g., those over 100 pS. The inactivation by drugs, e.g., cyclosporin and amiodarone (Antonenko *et al.*, 1991), is reminiscent of the action of the modulator protein on VDAC activity (Holden and Colombini, 1988).

At this time we cannot discriminate between a voltage-activated progressive increase in channel conductance due to an increase in limiting pore diameter or one in which low conductance units aggregate and open cooperatively in parallel. However, the frequent presence of a preliminary small increase in conductance preceding the larger increase and the similar event taking place during decrease in conductance (Fig. 1) suggest a complex pattern of cooperative behavior. The similar stepwise conductance decrease induced by inhibitors (Fig. 4) and increase induced by voltage (Figs 2 and 3) support a mechanism for activation and inactivation which could involve assembly and disassembly. Similar conductance changes have been reported in mouse liver mitoplast patches with changes in calcium on the matrix face of the inner membrane (Kinnally *et al.*, 1991) or the addition of the inhibitor amiodarone (Antonenko *et al.*, 1991). A cooperative model for Ca^{2+} activation is supported by the aggregation of IMM proteins induced by Ca^{2+} (Fagian *et al.*, 1990).

Thus far, Zorov *et al.* (1991) has resolved a mini-

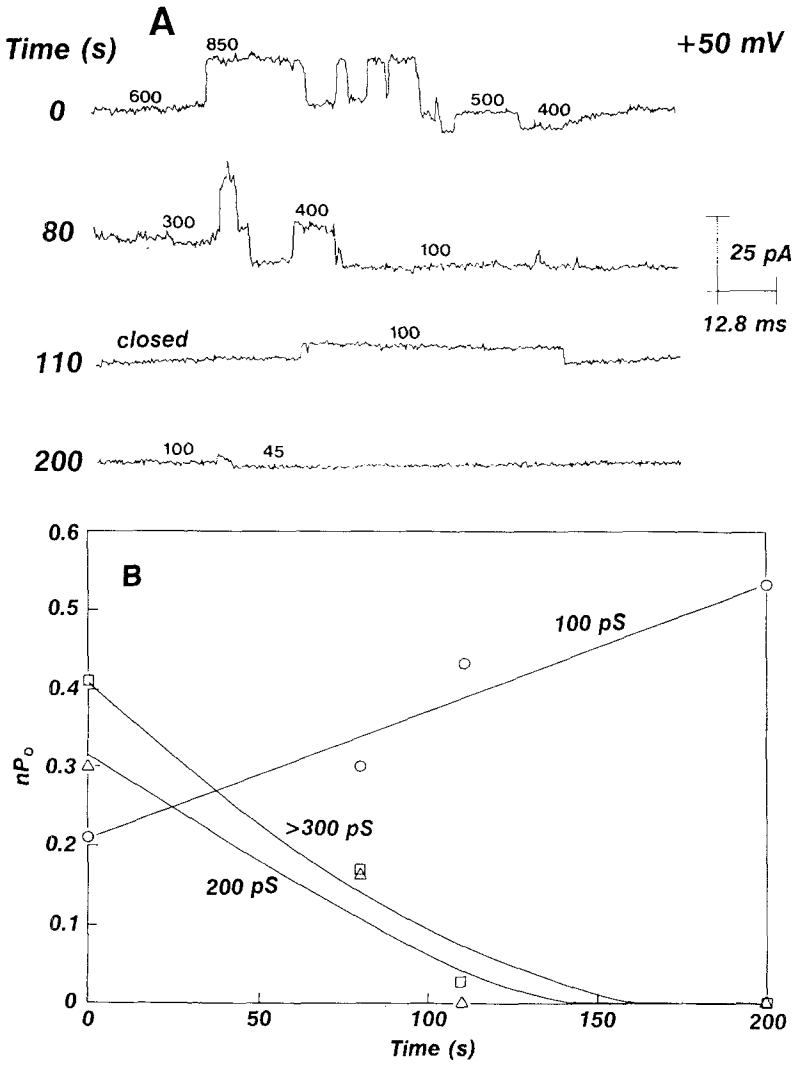

Fig. 4. Effect of 250 nM cyclosporin on the occupied current levels and transitions sizes. Conditions as in Fig. 2. A. Selected current traces at indicated time before, $t = 0$, and after, t indicated, cyclosporin addition at 4 kHz bandwidth. Numbers indicate the conductance level occupied and illustrate the loss of large transitions with time. B. The probability of opening, nP_O, to various conductance levels 100 pS (○), 200 pS (△), ⩾300 pS (□) after the addition of cyclosporin. Analysis at $t = 0$ s was for 60 s prior to addition, and for 30 s after each point shown using a bin width of 1 pA.

mum of nine subconductance states observed with MCC activity. While this is consistent with the existance of nine substates within a single channel, it could also suggest that a minimum of nine lower conducting units could be involved in an assembly to account for the cooperative behavior described. A maximal estimate of the number of units can be made by assuming the simplest assembly model and dividing the peak conductance (~ 1 nS) by the smallest resolved separation of conductance levels (20–40 pS). Such calculations suggest at least 25 units are involved. The cooperation of 9 to 25 parameters, e.g., by alignment of several dipoles, may be necessary to generate the nS transitions along with the multiple subconductance states described here. Mechanisms other than cooperative effects are also possible.

A model of cooperativity involving assembled units of lower conductance is not without precedent. A variety of investigators have proposed a similar mechanism to explain multiple conductance states in

other systems (reviewed by Meves and Nagy, 1989). These range from channels with two subunits (Miller, 1982) for the Cl^- channel of *Torpedo* electroplax, a four-barreled K^+-selective channel of *Amphiuma* kidney (Hunter and Giebisch, 1987) and a six-subunit anion-selective channel in alveolar epithelial cells (Krouse *et al.*, 1986). Clusters of 16 subunits were suggested by Geletyuk and Kazachenko (1985) for the Cl^- channel of molluscan neurons. Petronilli *et al.* (1989) also proposed a similar mechansim for the multiconductance channels of mouse liver mitochondria.

The physiological role of the inner mitochondrial membrane channels described in this study remains a subject for speculation. There are several possibilities. Amchenkova *et al.* (1988) have shown that mitochondria in cardiomyocytes are electrically coupled. These connections may correspond to the channel-like connecting structures resembling gap-junctions which were demonstrated by Bakeeva *et al.* (1983) in cardiomyocytes. Other possibilities are also still open. The channels could function in volume regulation (Gunter and Pfeiffer, 1990) by controlling the entry or exit of certain ions. Since the half-life of mitochondria is short (Fletcher and Sanadi, 1971, Luzikov, 1985), channels could also function in programmed mitochondrial destruction. Finally, a role for water-filled channels in mitochondrial protein import has also been suggested (Singer *et al.*, 1987).

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