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# Multiple conductance levels in rat heart inner mitochondrial membranes studied by patch clamping

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The behavior of the mitochondrial inner membrane multiple conductance channel (MCC) which has a peak conductance of 1–1.5 nS has been examined in rat heart mitochondria. MCC can display several unique characteristics: (a) prolonged open and closed times on the order of seconds to minutes, (b) a voltage dependence in which MCC opens (negative potential) or closes (positive potential) generally in steps, (c) a response to inhibitors such as amiodarone in steps corresponding at least approximately to those in (b), (d) a 'free-running mode' in which the current level rapidly fluctuates between a minimum of nine conductance levels but with a preferred occupation of the 0.5–0.7 nS levels, and (e) very large transitions (1–1.5 nS) resolved at 4 kHz bandwidth as single events with variable mean open time.

## Introduction

Patch clamping of the inner mitochondrial membrane (IMM) of mouse liver has revealed three kinds of channel behavior, (a) ≈ 110 pS voltage dependent channel activity with slight anion-selectivity [1,2], (b) multiple conductance channel (MCC) activity [3,4] which we presently classify as a single group since the various levels in general have similar properties (see Discussion and Ref. 5), and (c) ≈ 15 pS channel activated by alkaline pH [6] (for a review see Ref. 5). Variations in isolation procedures allow for selective channel activation such that some membrane-patch recordings have no channel activity or predominantly ≈ 110 pS or MCC activity [2].

The MCC activity displays several unique features including its enormous nS conductance which can occasionally be resolved as single steps [3]. It can be activated by $Ca^{2+}$ [2,7] or by membrane potentials above approx. ±60 mV [8]. During potential activation it frequently exhibits an increase in unit conductance which takes place in steps [8], whereas inhibitors often cause a progressive decrease in conductance again in steps [8]. After activation by either $Ca^{2+}$ or membrane potential, MCC exhibits several unique characteristics and we are reporting five of these for the first time (see below).

Mitochondrial membrane fractions enriched in contact sites [junctions between the IMM and the outer mitochondrial membrane (OMM)] exhibit MCC activity [9]. This observation suggests that contact sites contain channels responsible for MCC activity.

Cardiac mitochondria used in these experiments have interesting structural and functional features. For example, in situ clusters of mitochondria in cardiomyocytes have been shown to be structurally connected at contact sites reminiscent of gap junctions [10,11]. The functional continuity of mitochondria in clusters has been recently demonstrated in cardiomyocytes [12]. In these experiments, the fluorescence of ethylrhodamine in an entire mitochondrial cluster is quenched by laser irradiation of one component of the group. However, unconnected mitochondria appear unaffected. These observations suggest the presence of a communication system, possibly channels between the mitochondria forming a cluster. This system may also involve MCC since the pathway must involve a structure going through the IMM and the OMM.

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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## Methods

*Preparation of mitoplasts*

Rats of the Sprague-Dawley strain averaging between 200 and 300 g were used. All steps were carried out in the cold. A single rat heart was homogenized in 0.30 M sucrose (pH ≈ 7) using a glass and Teflon homogenizer of the Potter-Elvehjem type. Large mitochondria were isolated from the rat heart homogenates using the method previously described [13]. The mitochondria were then resuspended in approx. 2 ml of 0.30 M sucrose, 5 mM Hepes (pH 7.4). Mitoplasts were prepared by an osmotic swelling in which a 0.5 ml aliquot of the mitochondrial suspension was added to 9.5 ml 5 mM Hepes (pH 7.4) and kept on ice for 5 min. They were centrifuged at $2000 \times g$ for 5 min. The pellet was resuspended in 5 ml 0.30 osmolal sucrose, 5 mM Hepes, 0.1 mM $CaCl_2$, 1 mM EGTA (approx. $10^{-9}$ M free $Ca^{2+}$), pH 7.4 and kept on ice for 5 min. The preparation was then centrifuged at $2000 \times g$ for 5 min. This last step was omitted in some experiments and is referred to in this paper as an EGTA wash. The pellet was resuspended in 0.30 osmolal sucrose, 5 mM Hepes (pH 7.4). The yield of mitoplasts from a 0.5 ml aliquot was typically very small. The stock suspensions were kept on ice.

In a few experiments the mitoplasts were isolated using the French-press method of Decker and Greenawalt [14]. After the initial isolation of large mitochondria, the pellets were incubated in 15 ml of 460 mM mannitol, 140 mM sucrose, 10 mM Hepes (pH 7.4) for 10 to 15 min on ice and subjected to 2000 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 (pH 7.4). After standing on ice for 5 to 10 min they were centrifuged at $10000 \times g$ for 5 min and resuspended in 3 ml of 150 mM KCl, 5 mM Hepes (pH 7.4). We have not observed differences in the results obtained with mitoplasts prepared with the French press method and those prepared with the osmotic method. The experiments of Figs. 2 and 5 are the only ones reported here which used the French-press method.

*Patch clamping*

For patch clamping about 50 $\mu$l of suspension were placed on a slide. After several minutes the slide was perfused with the final medium and generally some mitoplasts were left attached to the slide in about 400 to 500 $\mu$l of medium. Unless otherwise stated, 150 mM KCl, 5 mM Hepes, 1 mM EGTA, 0.95 mM $CaCl_2$ (approx. $6 \cdot 10^{-7}$ M free $Ca^{2+}$), pH 7.4 at room temperature (20 to 25°C) was used. New mitoplast preparations were generally prepared from 0.5 ml aliquots of the original mitochondrial suspension approximately every 2 to 3 hours when osmotically treated mitoplasts were used. Excised patches were formed by lifting the pipette away from a mitoplast attached to the slide after a gigaseal was formed. Recordings from inside-out patches displayed the same voltage dependence as that from attached patches. Pipettes ranged in resistance between 20 and 40 M$\Omega$. The results are reported without correction for in-series resistance of the pipette. In all experiments the reference electrode consisted of a Ag-AgCl wire connected to the bath through a bridge containing the medium and 2% agar.

*Amiodarone treatment*

Amiodarone was purchased from Sigma Chemical Company. 4 $\mu$M amiodarone was added to a 1 ml chamber by perfusion with 5 ml of the usual medium containing the drug.

*Electronics*

The details have been previously presented [3]. Voltage-clamp conditions were obtained using a Dagan 8900 or 3900 (inside-out mode) patch-clamp amplifiers. Current and voltage were recorded in *Y-X* fashion on a storage oscilloscope (model 5111 with 5A19N and 5A21N amplifiers from Tektronix Inc., Beaverton, OR) while the voltage was varied manually. All voltages are referenced to the matrix of the mitoplast where $V = V_{bath} - V_{pipette}$. The current (bandwidth of 10 kHz) and voltage outputs were digitized with a Neurodata Instrument Corporation, model DR-284 (New York, NY) or Instrutech Corporation, model VR-10A (Elmont, NY) and recorded on VHS tape. The current traces and the *I-V* curve were subsequently analyzed at a bandwidth of 2 kHz except where otherwise stated (4–8 kHz) with a Frequency Devices (Haverhill, MA), model 902 low pass filter.

*Analysis of data*

The data were analyzed using IPROC software (courtesy of C. Lingle, Washington University, St. Louis, MO) and PAT program from Strathclyde Electrophysiological Data Analysis software (courtesy of J. Dempster, University of Strathclyde, UK). Variance of the current traces was determined with a 5 point moving average using the method described by Patlak [15]. Amplitude histograms were compiled using IPROC or PAT programs with a bin-width of 0.1 to 0.8 pA. Open probability ($nP_o$) for conductance levels was calculated from the ratio of the time at the corresponding current level/total time determined from amplitude histograms.

## Results

MCC activity appears after isolation of mitochondria in a $Ca^{2+}$-containing medium or after activation with voltages generally higher than ±60 mV [2,5].

After activation we observed a variety of MCC behavior which include (a) a 'butterfly' pattern, (b) free-running and (c) mixed mode which are separately described below. Shifts between behaviors occurs spontaneoulsy with the mixed pattern predominating.

*MCC controlled by membrane potential: the 'butterfly' pattern*

In one behavior pattern, the open and closed times were inordinately long (minutes) giving rise to current-voltage curves resembling a 'butterfly'. Once the open or closed state was attained, the current level often remained at that level until the voltage was varied. Frequently MCC opened at negative and closed at positive potentials. One such pattern is illustrated in the single channel current-voltage ($I$-$V$) curve of Fig. 1 where the sequence of events is indicated by the arrows (beginning at $-40$ mV). The opening and closing generally occurred at specific voltages and the total current changes were reproducible in magnitude within the same membrane patch. The current transition generally occurred in steps as illustrated in Fig. 2 for another experiment. The initial vertical lines (capacitance spikes) indicate the application of the voltage step which is maintained during the experimental period. An expansion of the time scale (Figs. 2B and D) shows that the shifts from higher to lower conductance levels induced by positive potentials (e.g. Fig. 2B) were faster than the shifts in the opposite direction induced by negative potentials (e.g. Fig. 2D). Generally we found closing to occur 2–8-times faster than opening ($n = 8$ randomly selected patches). After reaching a steady-state level, the same conductance was maintained for long periods of time (seconds and minutes), although there is evidence of channel activity of much lower amplitude. In summary, the butterfly pattern of MCC had a strong voltage dependence and the mean open and closed times were in seconds or even minutes.

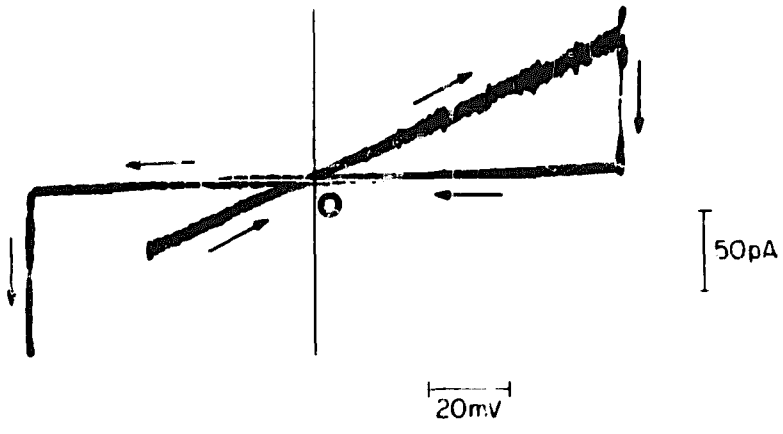



Fig. 1. One of the patterns of voltage dependence of MCC seen in a single channel current-voltage curve in an excised patch (butterfly pattern). The bath and pipette medium was 150 mM KCl, 1 mM EGTA, 0.95 mM $CaCl_2$ ($\approx 6\times10^{-7}$ M free $Ca^{2+}$), 5 mM Hepes at pH 7.4. The direction of the voltage changes (starting at approx. $-40$ mV) is indicated by the arrows. The voltage changes were controlled manually.

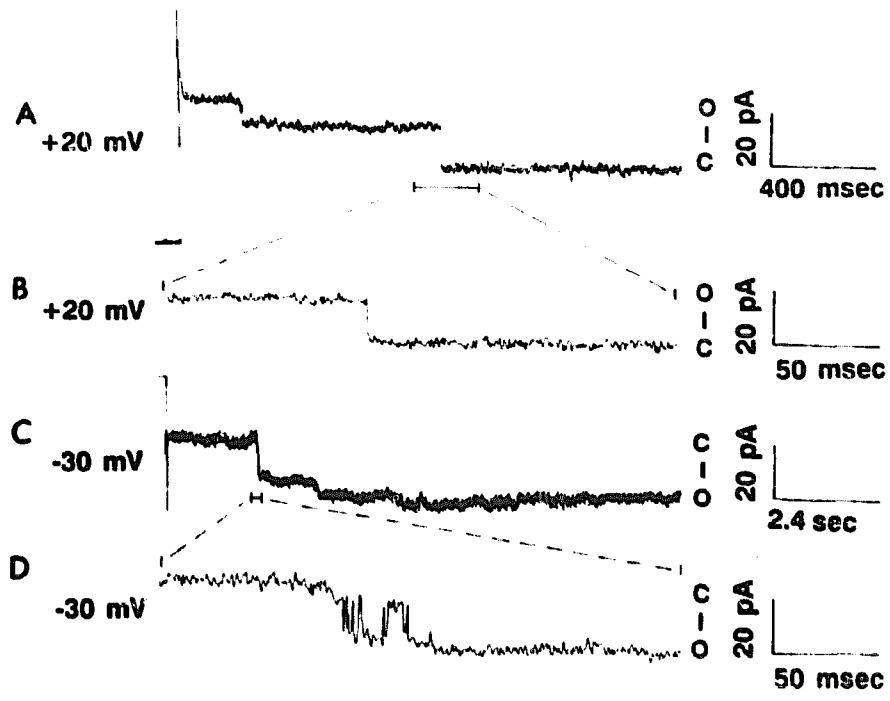



Fig. 2. Voltage steps for a butterfly type of patch (see Fig. 1) show fast closing (A,B) and slow opening (C,D). Conditions as in Fig. 1. Initial vertical lines indicate voltage on from zero mV to +20 mV (A) or $-30$ mV (C).

*Free-running mode*

MCC frequently exhibited a free-running pattern where a variety of conductance levels were observed. The traces of such activity are shown in Fig. 3A and the corresponding amplitude histogram is represented in Fig. 3B. A minimum of nine conductance levels can be recognized. The analysis of variance following the method of Patlak [15] (Fig. 3C), confirmed that these are discrete levels.

*Mixed pattern*

MCC most commonly was open at negative potentials and occupied lower peak and mean conductance at positive potentials. As MCC was open for prolonged periods at negative voltages, a constant high current level with no transitions was frequently recorded (see records at $-40$ and $-20$ mV, Fig. 4A and 4B). Upon shifting to increasing potentials, lower conductance levels were occupied as illustrated in the conductance amplitude histogram of Fig. 4B. The results summarized in Fig. 4C show the probability of being open for the 1.1 nS level decreased and a progressive shift to increased open probability of the lower conductance levels, i.e the 0.7 and 0.6 nS levels, was seen with increased voltage. While the prolonged open times at negative voltages resembled those of the butterfly pattern, the pattern resembled the 'free-running mode' in the positive range of potentials (e.g. Fig. 4A at +50 mV).

*Large conductance changes occurring as single events*

In some of the experiments single transitions as high as 1–1.5 nS were observed (e.g. Fig. 5) at a 4 kHz

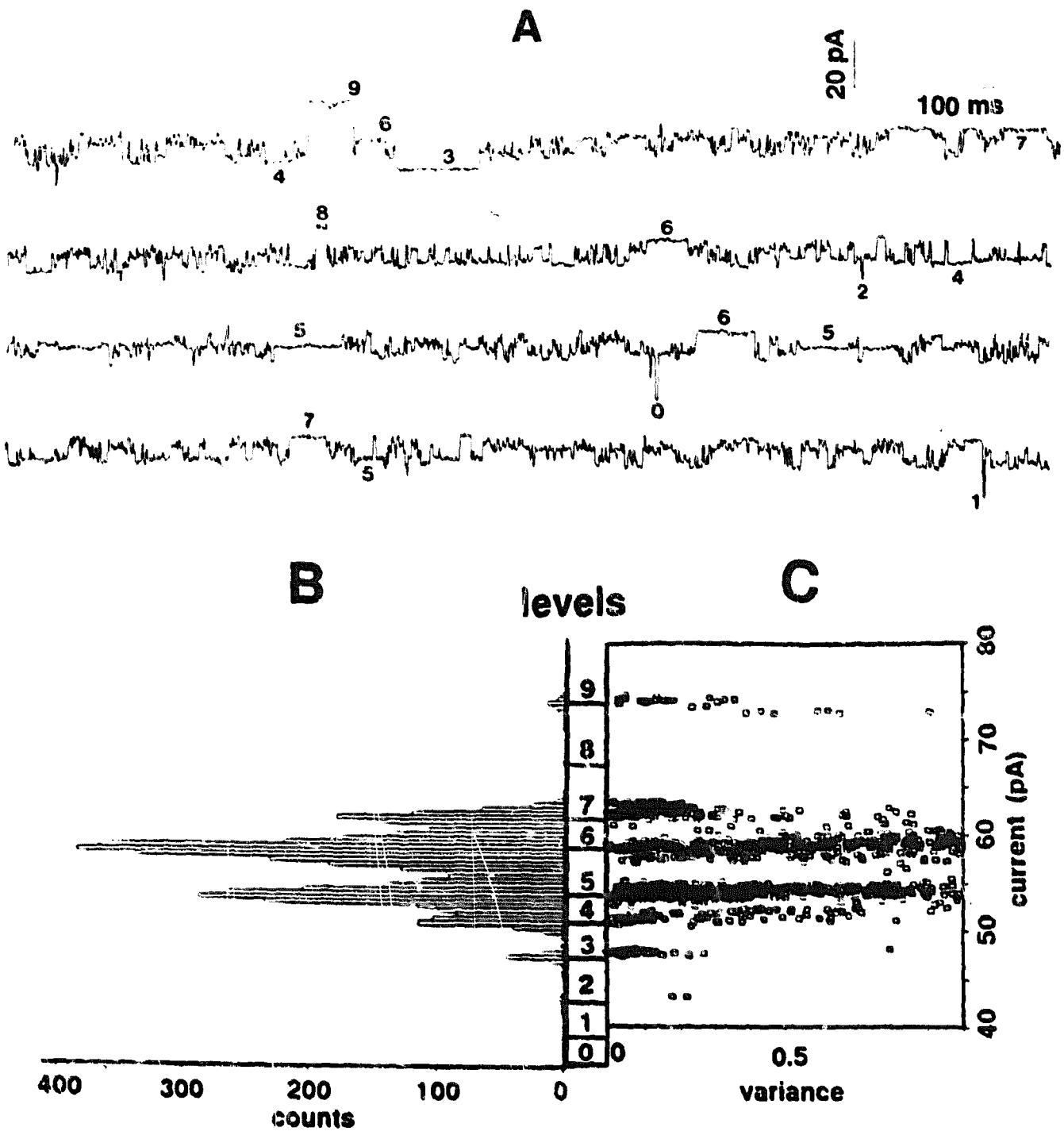


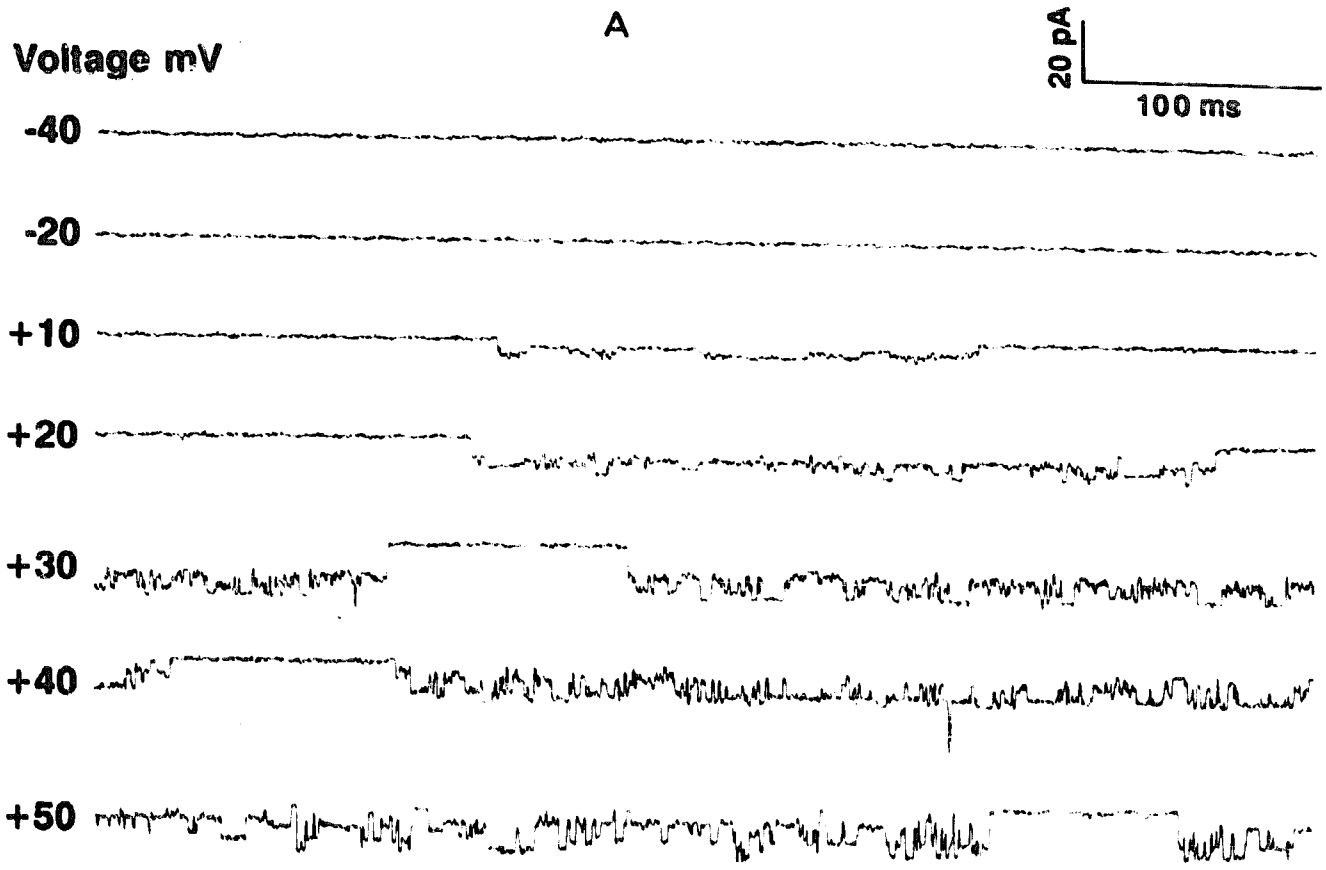

Fig. 3. Multiple conductance levels observed in free-running mode. Conditions as in Fig.1. (A) Selected sections of a current trace (512 ms each) at constant +40 mV show multiple conductance levels labeled from 0 to 9. The nine levels above the closed, 0, state indicated (from 1 to 9) in B and C correspond to approximately 0.08, 0.2, 0.3, 0.35, 0.45, 0.56, 0.7, 0.8 and 1.1 nS above the closed state. (B) Total amplitude histogram for 35 s showing occupancy of bins (0.4 pA width). (C) Mean-variance was computed for the current record collected over 1.75 s. See Methods for details.

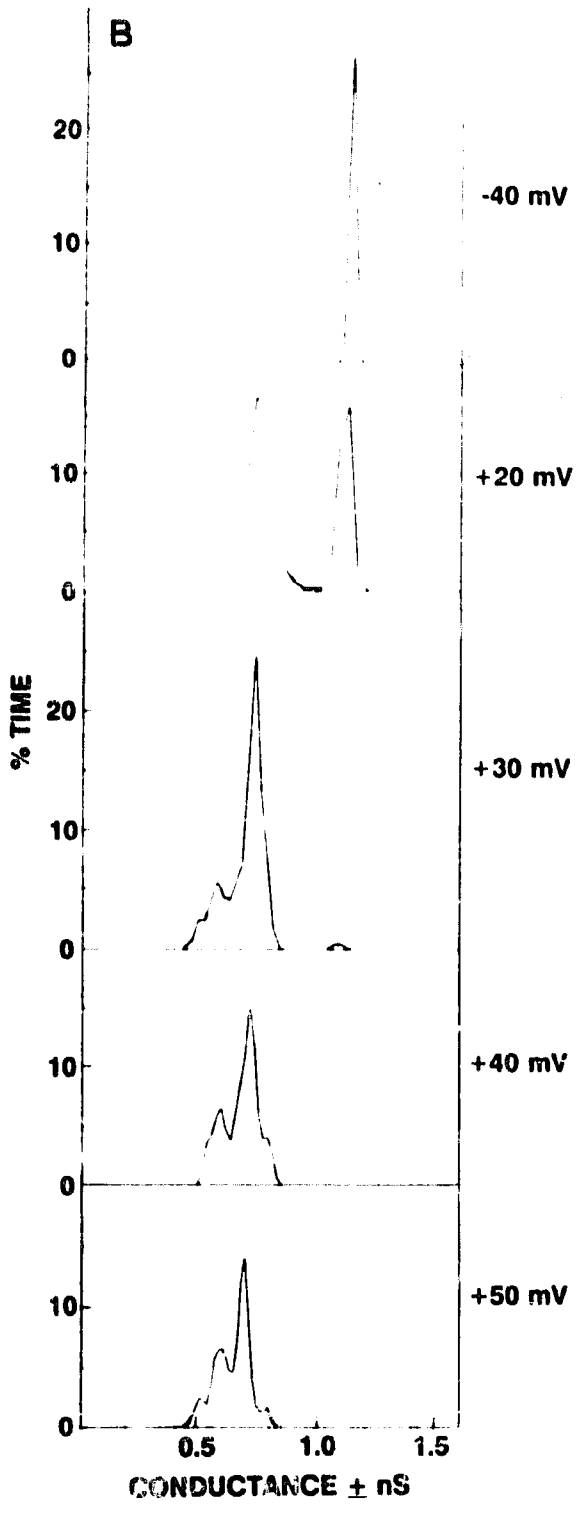
bandwidth under voltage clamp conditions. These may also occur repeatedly. Transitions of these sizes occur in 20% of the patches ($n = 25$ randomly selected patches) and were observed in butterfly, free-running and mixed behavior patterns.

*Inhibitors decreasing conductances in steps*

When MCC exhibited a butterfly pattern, the voltage-induced increase and decrease in conductance usually occurred in progressive steps where observed levels correspond to those observed in the free-running mode. Similarly when an inhibitor such as amiodarone was introduced the inhibition occurred in steps at least roughly corresponding to those observed during voltage-induced changes as shown in the current trace of Fig. 6A and the corresponding amplitude diagram of

Fig. 4. Mixed pattern voltage dependence of MCC is most frequently observed. Conditions as in Fig. 1. (A) Sample current traces are shown at various voltages to illustrate typical transitions. (B) Total amplitude diagram at selected voltages shows occupancy of conductance levels as % Time calculated from current levels and corrected for leak conductance using a bin width of 0.78 pA. (C) The probability of opening $nP_0$ as a function of voltage for the conductance levels at: 1.1 nS (□), 0.7 nS (+) and 0.5 nS (◇), are shown. The duration of trace analyzed at each voltage was generally 39 s.


A
Voltage mV
20 pA
100 ms
-40
-20
+10
+20
+30
+40
+50


B
-40 mV
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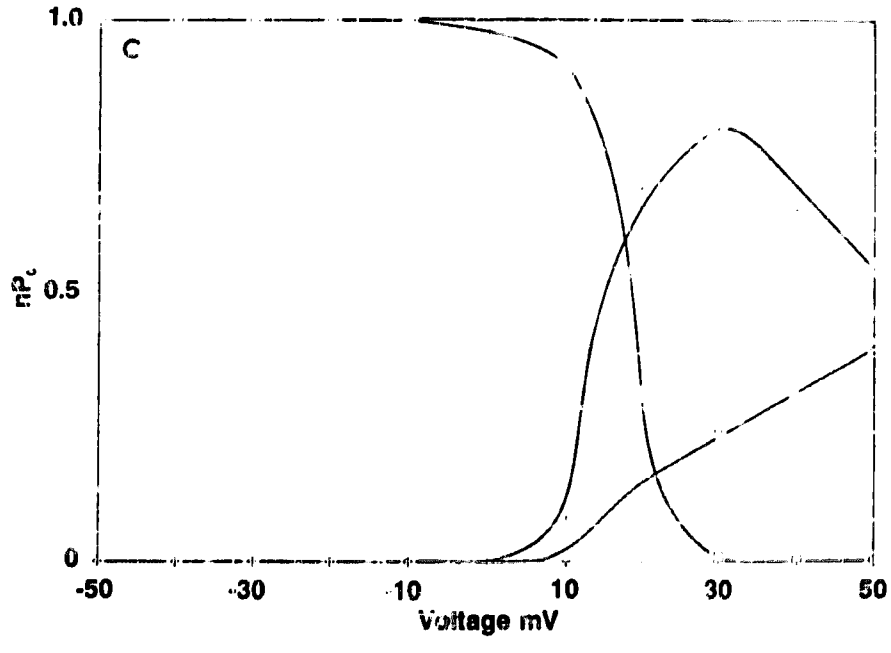
C
1.0
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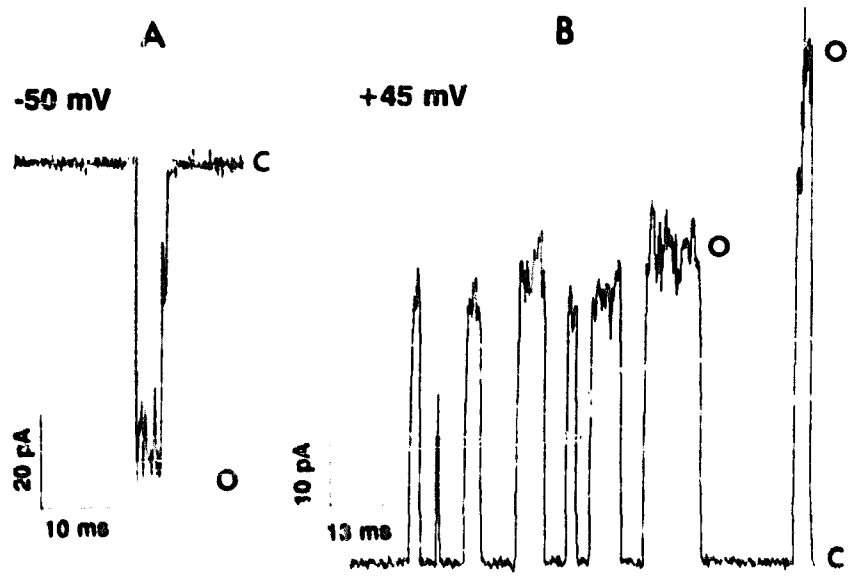


Fig. 5. Current traces show high conductance transitions at constant voltage with a bandwidth of 4 kHz (8 kHz sampling). (A) Continuous current trace at −50 mV shows transitions of 52 to 72 pA (1–1.4 nS). The bath medium was 150 mM KCl 1 mM EGTA 2 mM $CaCl_2$ (≈ 1 mM free $Ca^{2+}$) 5 mM Hepes (pH 7.4). The pipette medium was the same except the $CaCl_2$ was changed to 0.1 mM (free $Ca^{2+} \approx 10^{-9}$ M). (B) Current trace at +45 mV in a different patch showing transitions of up to 70 pA (1.5 nS) were observed. The medium in the bath and pipette was 150 mM KCl, 5 mM Hepes (pH 7.4).

Fig. 6B. We observed a similar inhibition in steps when MCC was blocked by cyclosporine [8] and antimycin A [16].

## Discussion

MCC activity has been observed by several investigators (for example, see Refs. 3 and 4). It appears to be highly regulated and opens only under special conditions [5]. Evidence has been presented indicating MCC may correspond to the $Ca^{2+}$-activated permeability transition pore (PTP) proposed from work with mitochondrial suspensions [5,17]. In our hands, either the presence of endogenous $Ca^{2+}$ during the isolation procedures or the exposure to voltages above ±60 mV are necessary to activate MCC [2]. Szabó and Zoratti have found MCC activity was activated by $Ca^{2+}$ and inhibited by $Mg^{2+}$, ADP and cyclosporine as expected for the PTP [17]. We consider the various conductance levels of MCC the result of the activity of one channel class since (a) they were most frequently observed in the same membrane patch [3,4], (b) they were activated by similar conditions [2,7,8], (c) they had a similar sensitivity to pharmacological agents [7,18], (d) the various levels were activated sequentially by voltage pulses [7], (e) current traces indicated that levels between 300 and 1300 pS were substates of a single channel since increases in conductance produced in small steps closed as a single event [4], and (f) the noise level of the fully closed and open state was generally lower than in the intermediate conductance levels [5]. The present study adds further support to the classification of at least nine conductance levels as substates of MCC. These transitions appear under the same conditions and the same patches as the nS transitions. In addition, the results cannot be explained by the presence of multiple copies of a single channel since the conductances are not multiples of a base conductance. Similarly some intermediate conductance levels are not present. Also, we were unable to fit (not shown) the asymmetrical amplitude diagrams with binomial equations describing independently identical channels (for example, see Ref. 25).The levels observed in response to voltage in the butterfly, free-running and mixed modes correspond to those observed with the addition of amiodarone [18], antimycin A [16] and cyclosporine [8]. These drugs mimic the effect of positive voltage in the butterfly behavior pattern in the induction of the closed state. Reconstitution of this activity with purified protein has not been done. Hence, the evidence is not conclusive and it is possible that more than one channel class is present under MCC-activating conditions.

The present study showed the presence of MCC in the IMM of heart mitochondria. Furthermore, the study defines for the first time several distinct and novel patterns of behavior. Generally two types of gating behavior were observed. In one MCC had long open and closed times. In another, the free-running behavior, MCC assumed many conductance levels in a short period of time (tens of seconds). As 'gearshifting' [19] between the two modes was occasionally observed and mixed behavior was most often observed, the shift in gating is reversible either spontaneously or with voltage. The inhibition with amiodarone below the lowest conductance level observed without the inhibitor suggests that some patches frequently have conductance states which are open indefinitely under the conditions of the experiments. Although we have not conducted a systematic study such as this in mouse liver mitochondria we have observed a similar behavior.

During voltage activation [8], the conductance progressively increased with time in steps. Similarly after activation, the inhibitor and voltage induced shifts in conductance between the various levels progress in steps. A model in which the increases in conductance result from assembly from subunits and conversely that decreases in conductance result from disassembly constitutes an attractive model that was originally put forth by Petronilli et al. [4] and is supported by the $Ca^{2+}$-induced aggregation of IMM proteins reported by Fagian et al. [20]. The higher conductance could be the product of an increase in pore diameter as we previously proposed for outer membrane channels [21,22] or from the various smaller channels opening and closing cooperatively. However, a model involving conformational changes leading to the progressive enlargement of a single channel pore diameter is equally

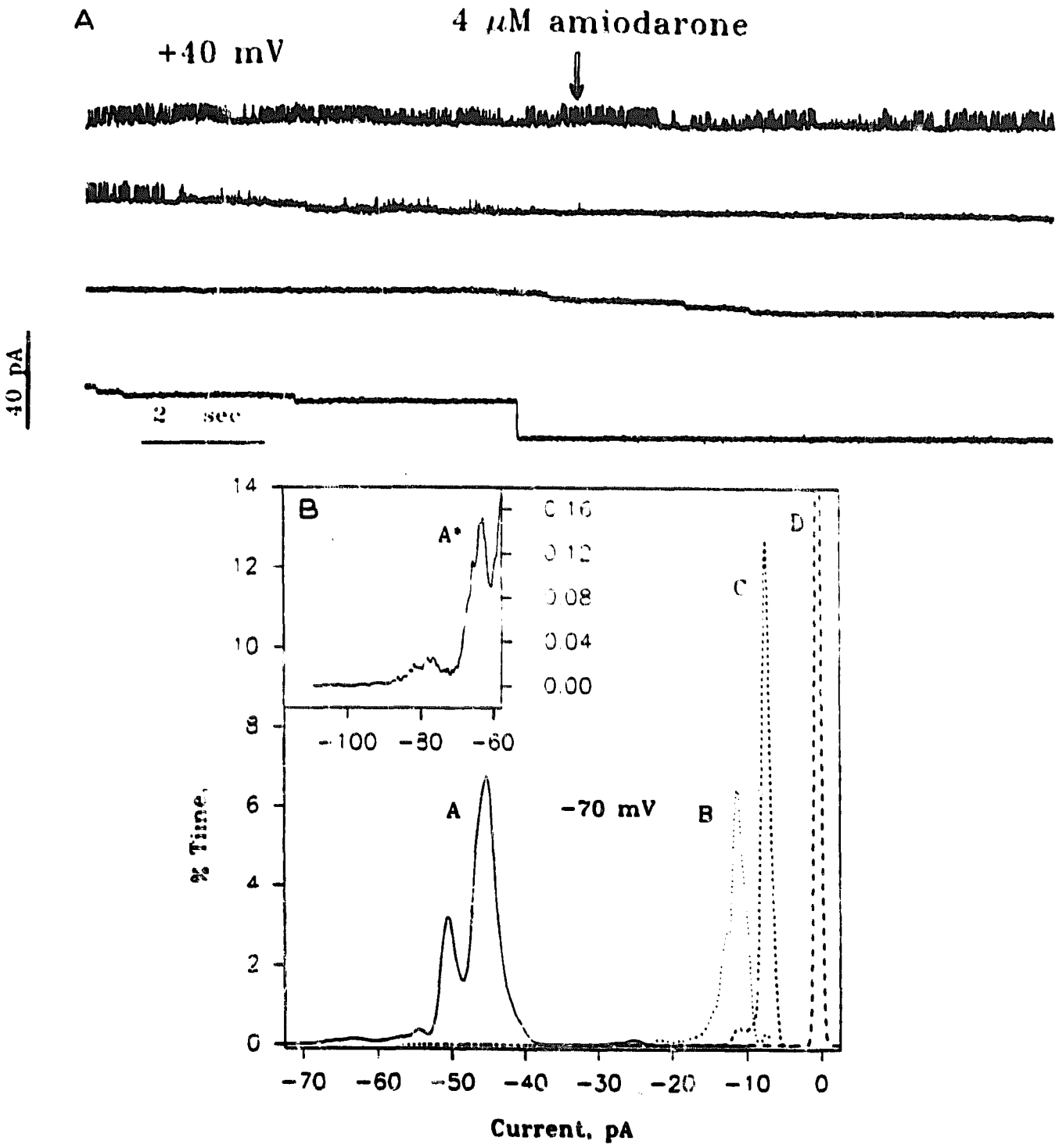


Fig. 6. Inhibition by amiodarone of the current through the patch. Medium as in Fig. 5A and other conditions as in Fig. 1. (A) Current trace at constant +40 mV showing the stepwise decrease in current after perfusion with amiodarone as indicated by arrow. (B) Amplitude histograms were constructed from data compiled from current traces with the following time intervals: A and A*, $t = -45$ to 0 s; B, $t = 75$ to 106 s; C, 106 to 127 s; D, 480 to 542 s. Amiodarone was added at zero time. The occupancy at each level is shown as % time which indicates the % of the total time spent at each current bin for each segment of the trace (i.e. A, B, C or D). Current bin size was 0.39 pA for A and 0.2 pA for B–D. A* insert illustrates the higher current levels of A with an expanded scale. Histogram peaks include conductance levels of approx. 0.1, 0.15, 0.2, 0.3, 0.6, 0.7 and 1.1 nS.

supported by the present data. Alternatively, a combination of both models cannot be eliminated. All the present results can be interpreted by postulating that each pattern of behavior reflect a difference in gating mechanism involving many (minimum nine) substates.

Multiple conductance states of a single channel is not without precedent. For example the OMM channel VDAC has been shown to have as many as five states [23]. The presence of multiple conductance states has also been shown in plasma membranes. Geletyuk and Kazachenko show evidence for ≈ 16 subconductance states (multiples of ≈ 12.5 pS each) of the $Cl^-$ channel for molluscan neurons and propose an assembly of channel subunits in clusters [24]. An anion-selective channel with six substates (integer multiples of 60–70 pS) that is usually fully open or fully closed has been described in epithelial cells [25]. Reconstitution experiments of gap junction protein also show multiple conductance levels of 140, 280 and 420 pS in 100 mM NaCl [26].

The variety of MCC activity modes is surprising. We have argued that IMM channels must be highly regulated [5], possibly by modulators similar to the protein shown to modify the activity of the voltage-dependent anion channel (VDAC) of the OMM [27,28]. Modulators which respond to subtle changes in conditions may also modify the behavior of MCC sufficiently to account for the varied patterns of behavior presented in this report. The possibility also remains that the various patterns represent specialized membrane locations,

such as contact sites between the IMM and the OMM or where mitochondria were originally in contact with each other.

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