In a patch-clamp study, we found antimycin A in low (1-2) nM concentrations decreased the open probability of the multiple conductance channel activity and the ~110 pS channel of the inner mitochondrial membrane (for a review of mitochondrial channels see Kinnally, K. W., Antonenko, Yu. N., and Zorov, D. B. (1992) J. Bioenerg. Biomembr. 24, 99-110). Higher antimycin A concentrations (e.g. 10 µM) facilitated multiple conductance channel opening. These effects were reversible, and the binding site(s) are probably distinct from those responsible for the inhibition of the electron transport chain, since the latter are virtually irreversible. A model with two closed and two open states is presented for the ~110-picosiemens activity.

Recent studies of the native inner mitochondrial membrane using patch-clamp techniques have identified three separate channel activities (for review, see Ref. 1). In 150 mM KCl, one of the activities has a strong voltage dependence and a conductance of ~110 pS (2) and another (MCC) displays multiple conductance levels with a peak of 1-1.5 nS (3, 4). In addition, a low conductance channel activity of about 15 pS (5) is induced by alkaline pH. Amphiphilic drugs decrease the probability of opening of the three kinds of channel activity and, at high enough concentrations, can block activity entirely (5, 6). In addition, amiodarone and propranolol increase the open probability of the 110 pS channel (6). Cyclosporine A and Mg²⁺ block MCC but not the 110 pS channel (7, 23). The differential effect of the drugs and Mg²⁺ suggests that at least MCC and the 110 pS channel activities are likely to correspond to two distinct channels (6, 7, 23).

This article presents data describing the effect of the respiratory inhibitor antimycin A (AA) on the MCC and 110 pS channel activities and presents possible mechanisms for AA action. A model summarizing the effects of voltage and AA on 110-pS activity is also presented.

MATERIALS AND METHODS

Large mitochondria were isolated from normal mice by a modification of the method previously presented (8). The homogenization medium was 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4, 1 mM EGTA (Medium I). After centrifugation at 110 x g for 80 s, 4 ml of supernatant were layered on 5 ml of 0.5 M sucrose, 5 mM HEPES, 1 mM EGTA, 0.95 mM CaCl₂ (pH 7.4), and centrifuged at 800 x g for 3 min. The mitochondria in the top layer and interface were then sedimented at 2,000 x g for 5 min and resuspended in 15 ml of the 460 mM mitochondrial, 140 mM sucrose, 10 mM HEPES, pH 7.4, and 1 mM EGTA, 0.95 mM CaCl₂ (pH 7.4). Mitoplasts were prepared using the French press procedure of Decker and Greenawalt (9) at 2,000 p.s.i. 10 ml of Medium I with 0.95 mM CaCl₂ was added to the mitoplasm suspension. After centrifugation at 2,600 g for 10 min, the mitoplast pellet was resuspended in 150 mM KCl, 5 mM HEPES, pH 7.4, 1 mM EGTA, 2 mM MgCl₂ (~24 µM free Mg²⁺), 0.1 mM CaCl₂ (~4 mM free Ca²⁺), and 2.5 µM rotenone (Medium II). No difference was detected when 230 mM mannitol, 70 mM sucrose replaced the 150 mM KCl as the resuspension medium. Excised patches were generally used, but AA was also effective in the attached patch configuration.

Experiments were carried out at ~25 °C in Medium II. Additions were made by a 5-μl perfusion of the 1-ml chamber. The order of additions did not change the results, as the effects were reversible. AA (Sigma, A 2006) was dissolved in ethyl alcohol or dimethyl sulfoxide with no detectable difference. Liposomes were prepared from purified soybean L-a-phosphatidylcholine (Sigma Type II-S) as previously described (22), using the method of Criado and Keller (16), except that ethylene glycol was omitted and ~10-µl aliquots of the small liposomes were used during the dehydration-hydration cycling.

Mitoplasts were examined under voltage clamp conditions, and the data were analyzed as previously presented (3) using a Dagan 3900 patch-clamp amplifier. Microelectrodes (World Precision Instruments, New Haven, CT; 1b100f-4) had a resistance of 20-40 MΩ. The data were recorded at 10 kHz, and analysis was usually bandwidth-limited to 2 kHz by an eight-pole (Frequency Devices, Haverhill, MA, model 902) low pass filter. The open probability, Pₒ, was computed from amplitude histograms as the ratio of percent time at open current level(s) over the total time. Histogram current levels are reported relative to closed current level. Membrane potentials (V) are reported relative to the mitochondrial matrix, i.e. V = Vₑlectrode - V_matrix.

RESULTS

The 110-pS and MCC activities were differentially activated by manipulation of calcium levels (1, 10) and applied voltages (1, 11). We found that 2 µM AA was generally sufficient to inhibit both channel activities in the voltage range ~250 mV, as illustrated in Fig. 1 for the 110 pS channel (20 mV) and Fig. 2 for MCC activity (30 mV). Figs. 1A and 2 are sample current traces, and Fig. 1, B and C, express the occupation of current levels in the form of amplitude histograms. Fig. 2 also shows the reversible MCC opening with high and closing with low concentrations of AA. The details of the observations on the two kinds of channel activity are presented separately below. In all cases, we found the effects of AA were completely or almost completely reversible by perfusion with fresh medium lacking the inhibitor. Frequently, a decrease in the closed channel current level was observed with AA, which may be due to inhibition of unre-
Antimycin A Affects Mitochondrial Channel Activity

**Fig. 1.** AA inhibits the 110-pS channel activity at 20 mV. A, sample current traces as indicated from recordings used to generate amplitude diagrams from a patch containing two active channels. Amplitude diagram shows occupancy of current level as per cent time (bin-width of 0.2 pA) for control (B) and in the presence of 2 μM AA (C). The closed channel current level arbitrarily reported as 0 corresponds to 5 pA in the presence of AA and 22 pA in the control. Seal resistance was ~4 GΩ. Similar results were obtained in 17 of 21 different patches.

**Fig. 2.** Effect of AA on MCC activity at 30 mV. Sample current traces as indicated were obtained sequentially. Inhibition of control activity by 2 μM AA was reversed by 10 μM AA. The opening seen with 10 μM AA was reversed with 1 μM AA. Seal resistance was ~10 GΩ. Similar results were obtained in 25 of 30 different patches.

**TABLE I**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Conductance (pS)</th>
<th>Voltage (mV)</th>
<th>pS</th>
<th>nP&lt;sub&gt;o&lt;/sub&gt;</th>
<th>Mean burst length</th>
<th>Openings per burst</th>
<th>Mean Openings</th>
<th>Mean Mean Openings</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>117</td>
<td>20</td>
<td>0</td>
<td>0.49 ± 3.7</td>
<td>8.9 ± 74</td>
<td>6</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>199 ± 3</td>
<td>30</td>
<td>0</td>
<td>0.76 ± 1.8</td>
<td>0.6 ± 91</td>
<td>21</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>110 ± 10</td>
<td>40</td>
<td>0</td>
<td>0.86 ± 5.9</td>
<td>0.9 ± 102</td>
<td>13</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>112 ± 1</td>
<td>50</td>
<td>0</td>
<td>0.94 ± 13.6</td>
<td>13.8 ± 408</td>
<td>23</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>199 ± 3</td>
<td>60</td>
<td>0</td>
<td>0.76 ± 2.1</td>
<td>2.1 ± 91</td>
<td>14</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>110 ± 10</td>
<td>70</td>
<td>0</td>
<td>0.86 ± 5.9</td>
<td>0.9 ± 102</td>
<td>13</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>112 ± 1</td>
<td>80</td>
<td>0</td>
<td>0.94 ± 13.6</td>
<td>13.8 ± 408</td>
<td>23</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>199 ± 3</td>
<td>90</td>
<td>0</td>
<td>0.76 ± 2.1</td>
<td>2.1 ± 91</td>
<td>14</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>9</td>
<td>110 ± 10</td>
<td>100</td>
<td>0</td>
<td>0.86 ± 5.9</td>
<td>0.9 ± 102</td>
<td>13</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>112 ± 1</td>
<td>110</td>
<td>0</td>
<td>0.94 ± 13.6</td>
<td>13.8 ± 408</td>
<td>23</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

**Fig. 3.** The effect of AA on the 110-pS channel activity. A, probability of opening (nP<sub>o</sub>) as a function of voltage for seven channels (determined from amplitude histograms at high positive voltage) present in this excised patch. See Fig. 1. The amplitude histograms used to calculate nP<sub>o</sub> represent the records of 47-s duration. Seal resistance was ~5 GΩ. At 40 mV with 0, 0.5, 1, 2, or 5 μl of ethanol/ml (corresponding to 0, 1, 2, 4, or 10 μM AA) control nP<sub>o</sub> were 1, 1, 0.9, 0.9, and 0.8 (not shown).

**Single channel characteristics of the 110-pS activity**

nP<sub>o</sub>, the mean probability of the channel being open, was percent time spent in the open state. Mean open and closed times were the mean duration of the open and closed state transition, respectively. Burst length was the duration of bursts with a 5-ms maximum closed interval and excluded single openings. Analysis was generally bandwidth-limited to 2 kHz, with a sampling of 125-250 μs. Settling time was three points, and the transition threshold was 50% of the open current transition size.
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AA or decreasing potential from ~50 to ~50 mV caused a progressive decrease in mean open time and an increase in mean closed time, as well as a decrease in burst length and open probability. Kitisch and Siemen (17) reported a decrease in the contribution of the slow components to the open time distributions, as the potential was changed from +50 to ~20 mV. Although we too observed an increase in the contribution of the fast components and a loss of slow components when the potential was changed from +50 to ~50 mV, similar effects were also observed upon the addition of AA (see Table II). The effect of AA on the single channel parameters was expected from the change in voltage sensitivity reflected in the shift of \( V_0 \).

Effects of Antimycin A on MCC—MCC activity displays many subconductance levels and has a variable, occasionally only slight, voltage dependence (1, 11). MCC was often open to 1–1.5 nS at negative potentials and had a lower open probability and occupied lower mean and peak conductances at low positive potentials (e.g. see Ref. 4). However, higher positive potentials (~50–150 mV) generally induced higher mean and peak currents and open probability, indicating a more complete opening. This voltage dependence of the open probability, \( nP_o \), is shown in Fig. 4A (control curve). Although the shape of these curves is often similar (reflecting the different voltage-dependent components), the potentials at which the different behaviors occur are variable from patch to patch. As previously reported (6), in some patches, the fully closed state was not observed unless a drug, e.g. amiodarone or AA, was added, regardless of voltage. AA at low concentrations (1–2 \( \mu \)M) decreased the open probability and mean current level in 25 of 30 independent experiments. There was a substantial block at concentrations as low as 1 \( \mu \)M (Fig. 4A). However, the inhibition could be overcome by potentials where negative potentials were generally more effective in opening MCC than were positive potentials (2 \( \mu \)M, Fig. 4A). Because of the complexity of analysis when many substates are present, the effect of AA on single channel parameters was not examined.

Above 2–4 \( \mu \)M, AA facilitated MCC opening at both positive and negative potentials, as illustrated in Fig. 4B, for 30 mV by an increase in open probability at 10 \( \mu \)M. The kinetics of this facilitated opening were examined, as shown in Fig. 5. In this experiment, MCC was closed at ~40 mV and opened rapidly at ~100 mV. To slow down the opening, the potential was stepped between ~40 and ~100 mV at 1 Hz to voltage induce MCC opening, as previously reported (11). While ~10 s was needed for opening in the absence of AA (indicated by an increase in mean current at ~100 mV), much longer (~75 s) was needed in the presence of 2 \( \mu \)M. However, at a higher concentration (e.g. 16 \( \mu \)M), the opening occurred within the perfusion period (Fig. 5).

### Table II

**r contribution (%) to open time distribution**

The sum of one, two, or three exponential probability density function(s) was chosen to fit histograms of dwell time using a Levenberg-Marquardt, nonlinear, least squares method and the percent area is listed as percent contribution. —, no contribution for that decay time constant. Data correspond to Table I, experiment 3. With some variability in numbers, the same trend of increasing contribution of fast components was observed with increasing AA concentration (\( n = 4 \) patches) and negative voltage (\( n = 6 \) patches).

<table>
<thead>
<tr>
<th>Voltage</th>
<th>[Antimycin A]</th>
<th>Decay time constants (( r ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( 0.8 \pm 0.2 ) ( n=4 )</td>
</tr>
<tr>
<td>~50</td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td>~20</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>~40</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>+10</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>+10</td>
<td>0</td>
<td>50</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Antimycin A Affects the Voltage Sensitivity of the 110-pS Activity—Previous studies of plasma membrane channel behavior indicated that voltage-dependent gating occurs in response to the presence of a voltage sensor, which allows the channel to respond to voltage (19). The shift in \( V_0 \) of Fig. 3 indicates that AA affected the closed state through the sensor, perhaps by altering the local environment, as was suggested in studies of the Na⁺ channel with the steroidal alkaloid toxin batrachotoxin (20). Either a progressive change in potential from ~50 mV to ~50 mV or increased AA concentrations at constant voltage caused similar changes in all the single channel parameters examined (see Tables I and II). Hence, the shift in \( V_0 \) induced by AA could account for the changes in the single channel parameters. The addition of 2 \( \mu \)M AA decreased the mean open time 75 ± 15% (mean ± S.D., \( n = 9 \) determinations on four different patches), regardless of voltage (between ~20 and 50 mV; see Table I). This is in contrast to the effect of the drug amiodarone (6), which did not affect mean open time. The difference in the effect of the two drugs could be explained by suggesting that amiodarone acts at a different site. In agreement with this idea, we previously suggested that amiodarone may stabilize a closed state (6).
behavior. Two closed states are indicated by the bursting potentials. Two open states of the same conductance are proposed because of kinetic data, as two exponentials were generated. The variability of the single channel parameters from patch to patch (see Table I) may be partially due to differences in \( V_{o} \). A naturally occurring heterogeneity in channel populations, possibly due to differences in fixed charges on or near the sensor, was thought to play a role in variation of \( V_{o} \) observed with Na\(^{+} \) channels (20). Alternatively, the presence or absence of natural modulators could account for the variability. A modulator protein is known to affect VDAC, the outer mitochondrial membrane channel (19). The kinetics of the voltage-induced opening are affected by AA. In Fig. 5, 2 \( \mu \)M AA prolonged, whereas 10 \( \mu \)M AA reduced, the time needed to open at -100 mV, as compared with the control. The interplay between inhibition and facilitation of voltage-induced opening is also shown by the shift in potential needed to open the channel with increasing AA concentrations (Fig. 4A). While 40-50 mV was needed to open MCC in the control or with 1 \( \mu \)M AA, MCC did not open even at 80 mV in the presence of 2 \( \mu \)M.

The inhibitory effect of AA could be overcome by potential, regardless of polarity, as shown in Fig. 4A. Negative potentials appear to be more effective, even though AA is not charged. A similar asymmetry was observed previously with the inhibition of MCC activity by some amphiphilic cations (amiodarone, quinine, and propranolol) (1) and for the initial voltage activation of MCC in patches in which initially no channel activity was recorded (10). These observations suggest that the mechanisms of these effects may have some common features.

The effect of AA on the activation by voltage of MCC suggests an involvement of a voltage sensor as we already proposed for the 110-pS channel and is thought to occur in other systems. The gating of some K\(^{+} \) channels is activated by Ca\(^{2+} \) binding, presumably by inducing conformational changes that stabilize the open states (21). Similarly, batrachotoxin activated Na\(^{+} \) channels in planar bilayers and nerve cells (e.g. 14, 15), probably by a multiplicity of effects, including blocking inactivation and affecting voltage sensitivity. Hence, the facilitation of MCC opening induced by higher concentrations of AA may be related to changes in voltage sensitivity.

Since the facilitation of opening of MCC generally required a higher concentration of AA than the inhibition (e.g. see Fig. 4B) and the time course of the two was also different (generally activation is faster, not shown), two different binding
sites may be responsible for the two opposite actions.

Does Antimycin A Act by Binding to the 12-kDa Protein?—
To our knowledge, this is the first demonstration of an inhibition of mitochondrial inner membrane channel activity by an inhibitor of mitochondrial function. AA is known to bind to a 12-kDa protein of complex III, as well as to other sites (12). This latter binding was originally thought to be nonspecific. The inhibition of MCC and the 110-pS activity by AA occurs at fairly low levels (1–2 μM). However, we strongly suspect that the binding site(s) do not correspond to the high affinity site (Kd = 0.02 μM) responsible for blocking electron transport (13), presumably on the 12-kDa polypeptide. The inhibition of electron transport could not be reversed by simple washing with an aqueous medium (12, 13), while the effects on the channel activity were reversible by perfusion. The effects were not likely the result of a nonspecific effect on membrane lipids, since we found no effect of AA on current records of excised patches from liposomes devoid of protein (not shown).

Conclusions—We found that the electron transport inhibitor AA was an inhibitor of the 110-pS activity, and the likely mechanism involves an effect on the closed state. AA had a dual effect on MCC activity, where low concentrations inhibited and higher levels increased MCC activity. Two separate sites for AA binding on MCC are probably involved for these opposite effects, with the inhibiting site having a higher binding affinity.

AA is slightly more effective in inhibiting MCC than the 110-pS activity in patches containing both activities (not shown). We have tested many drugs on both activities, and the inhibition (with the exception of cyclosporine) was relatively nonselective. This lack of specificity may be the result of homologous sites on the protein(s) responsible for activity. Alternatively the two activities may result from functional forms of the same protein. However, it is difficult to see how this premise can be tested rigorously without reconstitution of purified or partially purified systems.

Acknowledgments—We thank Charles Bowman, Charles Allen, and Michael Fejtl for critically reading this manuscript and Carmen Mannella for his helpful discussions.

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