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Minireview

Modulation of Inner Mitochondrial Membrane Channel Activity

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Three classes of inner mitochondrial membrane (IMM) channel activities have been defined by direct measurement of conductance levels in membranes with patch clamp techniques in 150 mM KCl. The "107 pS activity" is slightly anion selective and voltage dependent (open with matrix positive potentials). "Multiple conductance channel" (MCC) activity includes several levels from about 40 to over 1000 pS and can be activated by voltage or Ca^{2+}. MCC may be responsible for the Ca^{2+}-induced permeability transition observed with mitochondrial suspensions. A "low conductance channel" (LCC) is activated by alkaline pH and inhibited by Mg^{2+}. LCC has a unit conductance of about 15 pS and may correspond to the inner membrane anion channel, IMAC, which was proposed from results obtained from suspension studies. All of the IMM channels defined thus far appear to be highly regulated and have a low open probability under physiological conditions. A summary of what is known about IMM channel regulation and pharmacology is presented and possible physiological roles of these channels are discussed.

KEY WORDS: Mitochondria; channels; mitochondrial inner membrane; pharmacology; patch clamp; inner membrane anion channel; permeability transition pore.

INTRODUCTION

A high-resistance inner mitochondrial membrane (IMM) is thought to have a key role in energy transduction to maintain the electrochemical gradient needed for energy coupling. For this reason, the presence of ion channels in the IMM was thought to be incompatible with mitochondrial function. However, the channels now known to exist in the IMM appear to be highly regulated and their presence is not likely to be germane to the question of mitochondrial coupling under normal physiological conditions.

For many years, evidence for channels in the IMM has been accumulating from a wide variety of studies done with mitochondrial suspensions. For example, a pH-activated anion channel was proposed by Selwyn *et al.* (1979) and further characterized by others (for a review see Beavis, 1992). In addition, a nonspecific pore is thought to be involved in a large permeability transition that is activated by Ca^{2+} (Hunter *et al.*, 1976; for a review see Gunter and Pfeiffer, 1990). Also, channels at contact sites (junctions between the outer and inner membranes) may be involved in protein import from the cytoplasm to the matrix (Schwaiger *et al.*, 1987).

Despite this growing indirect evidence, definitive proof of the existence of large ion-conducting pathways in the IMM has been observed only recently. The breakthrough has come with the application of "patch-clamp" techniques to the native IMM (mitoplasts) (Sorgato *et al.*, 1987; Kinnally *et al.*, 1989; Petronilli *et al.*, 1989) and reconstituted systems (Sorgato *et al.*, 1989; Moran *et al.*, 1990).

Thus far, patch clamp studies on native IMM (in

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Table I. Summary of Channel Classes

Class	Size (pS)	~ Density #/mitochondrion[a]	Voltage dependence	Effectors	Probable counterparts
Multi-conductance (MCC)	~40 to >1000	100–200	slight	Ca^{2+}, voltage, Mg^{2+}, ADP[d]	MMC[b] PTP[c]
107 pS	107	200–400	yes	Ca^{2+}, voltage	IMM[e] channel
Low conductance (LCC)	~15	400–800	no	pH, Mg^{2+}	IMAC[f]

[a]Assuming 3 μm mitochondrial diameter and 0.5 μm pipette diameter.
[b]MMC, mitochondrial megachannel (Szabó and Zoratti, 1991, 1992).
[c]PTP, permeability transition pore.
[d]From Szabó and Zoratti (1992).
[e]IMM, inner mitochondrial membrane (Sorgato *et al.*, 1987).
[f]IMAC, inner membrane anion channel (Garlid and Beavis, 1986).

0.15 M KCl) have resolved three different classes of channels whose general characteristics are summarized in Table I. In addition, electrophysiological studies with purified or partially purified mitochondrial proteins in reconstituted systems suggest the presence of additional channels (e.g., Mironova *et al.*, 1981, 1982, Costa *et al.*, 1991). Sorgato *et al.* (1987) first described a channel with a unit conductance of 107 pS and slight anion selectivity. The probability of being open for this channel increases when the matrix potential is positive. A second class of IMM channel activity was then described by Kinnally *et al.* (1989) and Petronilli *et al.* (1989). It is characterized by a very high peak conductance (1–1.5 nS) and multiple conductance levels. Hence, it was named "multiple conductance channel" (MCC) (Kinnally *et al.*, 1991). A third class of IMM channel activity has been recently reported by Antonenko *et al.* (1991a). It exhibits small current transitions and is activated at alkaline pH. It is named low-conductance channel (LCC) because the unit conductance is ~15 pS, much lower than the other two IMM channel classes. Inoue *et al.* (1991) recently described a low-conductance channel (~10 pS in 100 mM salt) which is cation selective and ATP sensitive.

We are engaged in a systematic study of IMM channel activities under a variety of conditions including voltage, divalent cations, pH, and a number of pharmacological agents. The results summarized in this review indicate that the IMM channels are highly regulated by a variety of modulators. A tentative model is presented below for each channel along with a discussion of possible physiological roles.

TECHNIQUES

After mitochondrial isolation, the outer membrane is removed to expose the inner membrane and the result is a mitoplast preparation. This is generally done either osmotically or by a method employing a French press (Decker and Greenawalt, 1977), although alternative means are available. A glass micropipette then seals to the IMM either spontaneously or after a slight negative pressure is applied. The current transitions observed are characteristic of channel class under voltage clamp conditions and are used to calculate conductance. All voltages are reported relative to the matrix ($V = V_{bath} - V_{pipette}$).

We use mainly two patch configurations, attached and excised. A patch is attached if the mitoplast is intact at the pipette tip. The membrane patch is excised by pulling the pipette away from the mitoplast attached to the slide. In excised patches, the matrix face of the inner membrane is exposed to the bath while the outer face is exposed to the pipette solution. Hence, perfusion of the matrix face can be done by changing the bath solution. The basic medium is 150 mM KCl and 5 mM HEPES, pH 7.4.

COEXISTENCE OF THE THREE CLASSES OF CHANNELS IN THE IMM

Each of the channel activities summarized in Table I is associated with the inner membrane. The 107 pS activity is the dominant activity reported when an IMM fraction is reconstituted in liposomes (Moran *et al.*, 1990). As shown in Fig. 1, 107 pS activity

Fig. 1. Coexistence of MCC and 107 pS in IMM patches from mouse liver mitoplasts. Current trace from an excised mitoplast patch in symmetrical 0.15 M KCl, 5 mM HEPES, pH 7.4, 1 mM EGTA, and 0.95 mM $CaCl_2$ ($\sim 6 \times 10^{-7}$ M free Ca^{2+}) shows transitions corresponding to both MCC (~ 1 nS) and 107 pS (110 pS) activity at +30 mV. Bandwidth was limited to 2 kHz. Mitoplasts were washed with 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, and 1 mM EGTA, pH 7.4.

(known to be associated with IMM) and MCC activity can be observed in the same membrane patch (Kinnally *et al.*, 1991). With some isolation procedures, over 50% of the patches reveal both activities simultaneously (Kinnally *et al.*, 1991). Similarly, MCC activity is often observed in patches containing LCC (not shown). A review of the conductances seen in several reconstituted IMM fractions is given by Moran and Sorgato (1992).

MCC ACTIVITY

We group many different conductance levels together as a single class called multiple conductance channel (MCC) activity in Table I. Frequently observed levels are at 30–50, 80–100, 140–180, 250, 350, 450–550, 650–750, 900–1000, and 1250–1500 pS. While these may correspond to more than one channel, at this time we define them as a single class because (a) they are frequently observed in the same patch (Kinnally *et al.*, 1989, Petronilli *et al.*, 1989), (b) they are activated by similar conditions, e.g., Ca^{2+} level (Kinnally *et al.*, 1991; Szabó and Zoratti, 1992), (c) they display similar pharmacological responses (Antonenko *et al.*, 1991b; Szabó and Zoratti, 1991), and (d) they are observed during the voltage activation of nS transitions (see below and Zorov *et al.*, 1992). In addition, the current traces of Petronilli *et al.* (1989) indicate that levels between 300 and 1300 are substates of a single channel as small openings are closed by a single larger step. There have been many reports of multiple conductance level channels in the plasma membrane (e.g., Krouse *et al.*, 1986 and Meeves and Nagey, 1989), and VDAC, the outer membrane channel, has multiple substates (Mirazabekov and Ermishkin, 1989).

The very high conductance states (> 900 pS) are often occupied for long periods of time (seconds to minutes) especially at negative voltages. As shown in Fig. 2, the fully open ($\sim$ 1000 pS) and closed levels are relatively low-noise states, while the intermediate levels often have a shorter mean open time and, higher current fluctuations are observed. This behavior is characteristic of channels with substates (e.g., Sachs, 1983).

Calcium Regulation of MCC

Calcium is known to induce a large increase in mitochondrial permeability (Hunter *et al.*, 1976 and Gunter and Pfeiffer, 1990). In electrophysiological studies, MCC activity is recorded from over 95% of the mitoplast patches when a Ca^{2+} chelator is omitted during mitochondrial isolation (Kinnally *et al.*, 1991). In contrast, chelation of Ca^{2+} during isolation results in recordings with no activity at low voltages (see below) over 70% of the time. The activation of MCC by Ca^{2+} is not reversible since the activity is still present in over 90% of the patches after EGTA washing of the mitoplasts prepared without chelator. Increasing free Ca^{2+} levels on the matrix face from 10^{-9} to 10^{-5} M results in a decrease in mean conductance. In contrast, Szabó and Zoratti (1992) found that mM Ca^{2+} increases the open probability of MCC (which was previously electrically silent) and reverse this effect with addition of EGTA. This latter finding and our results indicate that the Ca^{2+} effect is likely to be complex and involve several as yet unknown factors (see Discussion).

Voltage-Induced Cooperativity in MCC

Typically MCC displays three kinds of behavior. One state corresponds to a closed (inactive) state achieved by isolation in the presence of chelator and maintained by voltages between $\sim \pm 60$ mV (Kinnally *et al.*, 1991). The other two types are activated and observed when isolation is done in the absence of Ca^{2+} chelators or, alternatively, after voltage activation from the closed state (see below). Most commonly,

Fig. 2. MCC has multiple conductance levels. Current trace from excised mitoplast patch, where O and C correspond to open and closed states at + 80 mV, show lower noise level in fully open and closed states. Other conditions as in Fig. 1.

activated MCC is open at all voltages, although the peak conductance is somewhat lower at positive voltages (e.g., see Fig. 4A, below). Alternatively, MCC remains in a fully open state for extended periods of time (seconds or minutes) with little or no effect of voltage.

Voltage activation of the closed state generally occurs if the magnitude of the potential is greater than ± 60 mV. Once completely activated, MCC activity is observed even at low voltages (Zorov *et al.*, 1992). Figure 3 illustrates the voltage activation. After the application of a potential at zero time, the transition size increases in steps until the fully open state (~ 1000 pS) is attained. The rate of development of large transitions increases with increasing magnitude of voltage regardless of polarity (Zorov *et al.*, 1992). A possible interpretation of such data is that the large transitions (nS) associated with MCC activity are the result of a voltage-induced cooperativity of lower conductance units (Zorov *et al.*, 1992). Alternatively, it may be due to an increase in effective pore diameter. A Ca^{2+}-induced association of IMM proteins (Fagian *et al.*, 1990) suggests the involvement of an aggregation of subunits into a larger channel.

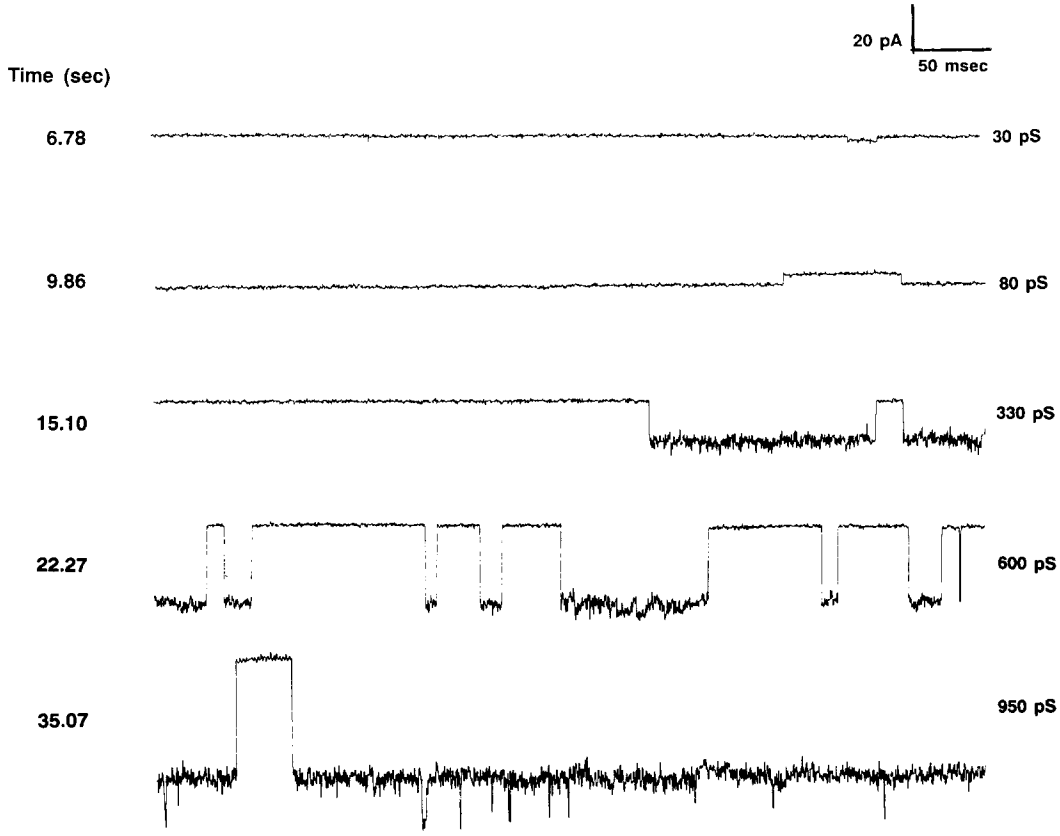

Fig. 3. Progressive activation of MCC by voltage. Current trace segments at times indicated of attached mitoplast patch at − 60 mV. Media was symmetrical 0.15 M KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.95 mM $CaCl_2$, and 5 mM HEPES, pH 7.2. Mitochondria were isolated in 230 mM mannitol, 70 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA, 0.95 mM $CaCl_2$, and 5 mM HEPES, pH 7.2. Other conditions as in Fig. 1.

Table II. Pharmacology of IMM Channels

Drug	MCC[a]	107 pS	LCC
Amphiphilic cations:			
Amiodarone (4 μM)	+	+	+
Propranolol (70 μM)	+	+	+
Quinine (0.6 mM)	+	+	+
Cyclosporine A (250 nM)[b]	+	−	n.t.[c]
Antimycin A (2 μM)	+	+	n.t.
Tributyltin (100 μM)	n.t.	n.t.	+

[a]At voltages between ± 60 mV.
[b]Szabó and Zoratti (1991).
[c]n.t.: not tested; concentration for complete inhibition in at least 70% of patches.

Pharmacology of MCC

By testing a large number of drugs known to affect IMM permeability (e.g., Beavis, 1989), we found several inhibitors of MCC activity (Table II). Cationic amphiphiles, including amiodarone, quinine, and propranolol, are among the first class of compounds found to be inhibitory (Antonenko *et al.*, 1991b). The current amplitude diagrams of Fig. 4A and 4B illustrate the decrease in conductance of MCC activity caused by quinine (see below). Recently, cyclosporine A was found to be a specific inhibitor of MCC activity by Szabó and Zoratti (1991). We also found antimycin A to be an MCC inhibitor (Campo *et al.*, 1990). Each drug often caused a stepwise decrease in mean conductance allowing visualization of levels that normally have a low occupancy. Once again, the conductance levels observed are the same as those observed at other times, e.g., during voltage activation (Zorov *et al.*, 1991).

While activated MCC shows only a slight voltage dependence (Fig. 4A), one of the effects of inhibitors is to establish a strong voltage dependence as seen in Fig. 4C. The closed state is not observed in the control and hence the open probability is 1 regardless of voltage. MCC is closed between -40 and $+80$ mV as indicated by an open probability of 0 after addition of 140 μM quinine. The effectiveness of the inhibitors is decreased by high voltage regardless of polarity (see Fig. 4C). A higher concentration of quinine is necessary to inhibit MCC at -80 mV than $+80$ mV, again indicating that negative voltage is more effective in overcoming the inhibition than positive voltage, as seen in Fig. 4D. This may be due to electrophoresis of the cationic drugs away from the patch with negative (pipette positive) voltages. However, preliminary results with antimycin A, which is not charged, showed similar patterns (not shown). The voltage effects on inhibited MCC are similar to that of inactive MCC. That is, sufficient voltage of either polarity can restore the activity of inhibited MCC (Zovov *et al.*, 1992). A possible interpretation is that the inhibitors may be restoring the inactive state in the same way as Koenig's polyanion is thought to replace the natural modulator for VDAC (e.g. Tedeschi *et al.*, 1987).

MCC Activity Model

MCC is shown as a two-state model, closed (or inactive) and open (active) in Fig. 5. For simplicity a single open state is shown. MCC can be activated from the closed to open state by high voltage or the presence of calcium during isolation. Once activated, MCC typically remains in an open state (Fig. 4A) and is weakly voltage dependent, i.e., occupying lower conductance levels with positive potentials. Occupation of the closed state is favored by a variety of drugs and observed if calcium is chelated during mitoplast preparation. Lower conductance levels are favored by elevated matrix calcium levels ($\geq 10^{-5}$ M).

We are proposing that MCC is associated with contact sites when calcium is present during mitoplast preparation as illustrated in Fig. 5A. This association is based on (1) reconstitution studies (Moran *et al.*, 1990) where MCC activity is observed in liposomes enriched in contact sites; (2) Ca^{2+} dependence of the integrity of contact sites (Sandri *et al.*, 1988); (3) the calcium-dependent activation of MCC during preparation (Kinnally *et al.*, 1991). In addition, electron micrographs of mitoplasts show that fragments of outer membrane are present at contact sites in some mitoplasts (not shown). Electrical continuity between the matrix and cytoplasmic sides of the mitochondrion at contact sites would require channels in tandem through both the inner and outer membranes and this is illustrated by the model of Fig. 5A (see also Benz and Brdiczka, 1992). The second form of MCC, shown in Fig. 5B, would be present at sites other than contact sites and could be activated by voltage (e.g., after isolation in the presence of a chelator). We have not detected differences in the open state of the two kinds of MCC.

We propose that the enormous peak transition size of MCC is the result of a cooperativity between lower conductance units induced by voltage or transient high Ca^{2+} levels during mitochondrial isolation. This cooperativity can be decreased by Ca^{2+} at the

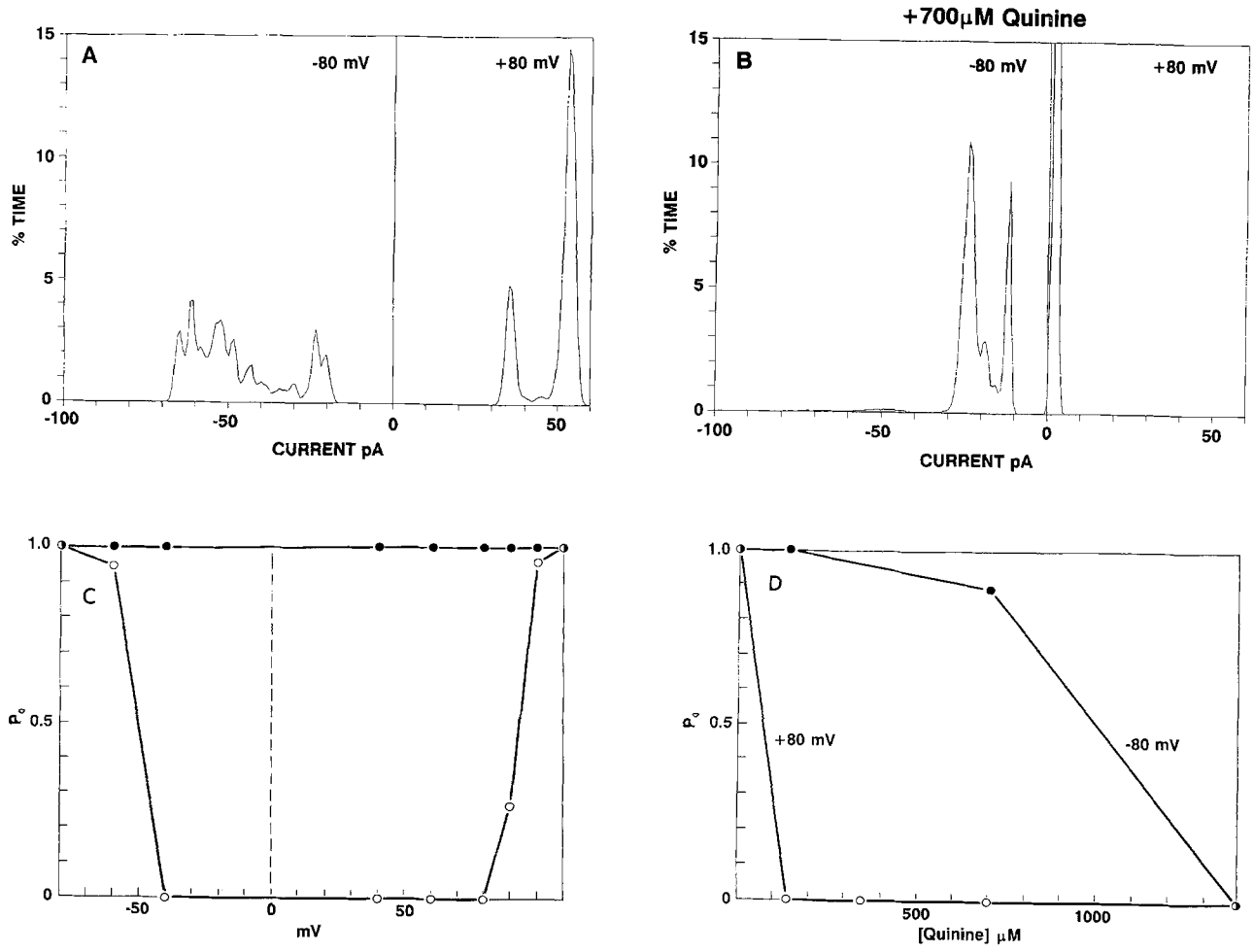

Fig. 4. The effect of quinine on MCC activity. Amplitude diagrams showing occupancy of current levels as percent time in the absence (A) and presence (B) of 700 μM quinine at ± 80 mV. With quinine, MCC was fully closed at $+80$ mV but only partially closed at -80 mV. (C) The voltage dependence of the open probability (P_O) of MCC. The percent time occupancies of all levels above the fully closed states were summed and divided by the total time to determine the open probability at each voltage in the presence (○) or absence (●) of 140 μM quinine. (D) The effect of quinine concentration on the open probability at ± 80 mV. Other conditions were as in Fig. 1.

matrix face or the addition of an inhibitor. However, at this time the data are equally consistent with a model in which the effective pore diameter changes. The multiple conductance levels indicate the existence of many substates not illustrated in Fig. 5.

Mitochondrial suspension studies suggest that a nonspecific pore is involved in a large permeability transition (for review see Gunter and Pfeiffer, 1990). MCC is probably responsible for the permeability transition as MCC is (1) Ca^{2+} activated (Kinnally *et al.*, 1991; Szabó and Zoratti, 1992), (2) inhibited by cyclosporine A (Szabó and Zoratti, 1991), (3) inhibited by ADP and Mg^{2+} (Szabó and Zoratti, 1992), and (4) relatively nonselective (Szabó and Zoratti, 1992, Kinnally *et al.*, 1989). See Szabó and Zoratti (1992) for further discussion.

As the intracellular Ca^{2+} levels *in vivo* are low and matrix ADP levels are high, MCC would not likely be activated under normal conditions. However, transient variations in cytoplasmic Ca^{2+} induced, for example, by hormones (Gunter and Pfeiffer, 1990) or ischemia may result in an activation of this channel. Further study of MCC regulation should provide insight into its physiological role (see Discussion).

107-pS ACTIVITY

The 107 pS activity was first described by Sorgato

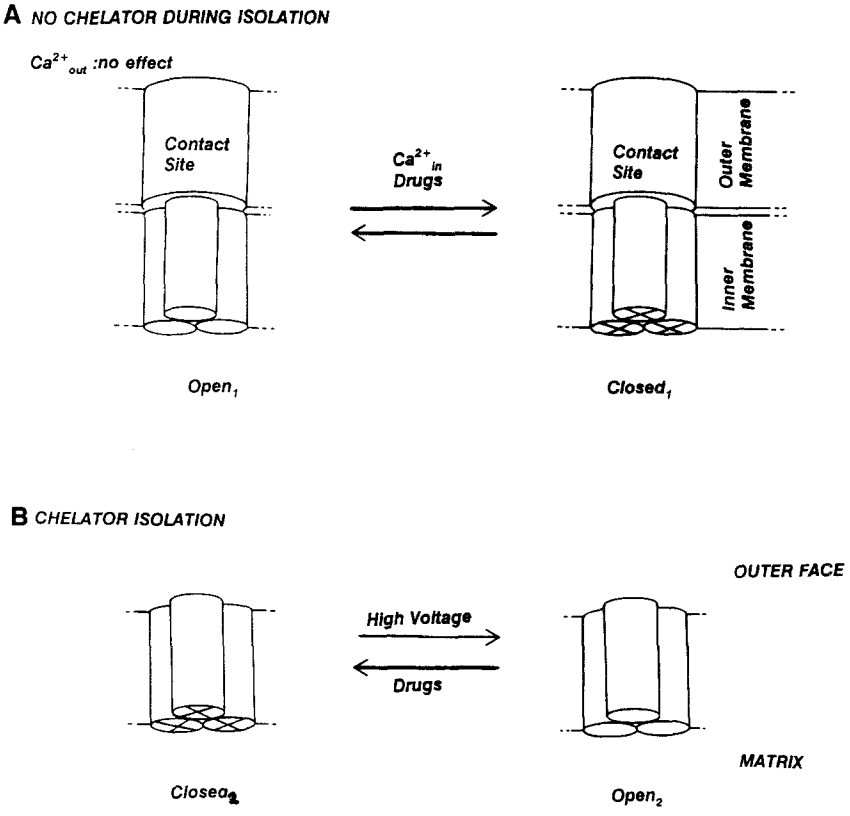

Fig. 5. Models for MCC activity. A: MCC associated with contact sites is active upon patching. Ca^{2+}_{out} and Ca^{2+}_{in} refer to Ca^{2+} above physiological levels in the pipette (out) or bath (in). B: MCC is initially inactive and may be located outside of contact sites. High voltage refers to magnitudes greater than ± 60 mV. Drugs are those indicated in Table II.

et al. (1987) and is further discussed in Moran and Sorgato (1992). It is voltage dependent (opening with matrix positive voltage) and slightly anion selective but not pH dependent (Table I). This activity is observed in less than 5% of patches if Ca^{2+} is not chelated during mitochondrial isolation (Kinnally *et al.*, 1991). If mitoplasts are washed with EGTA, the activity is seen in 65–70% of the patches. The effect is not reversible as the addition of up to 10^{-5} M free Ca^{2+} to the outer face does not eliminate this activity. The irreversibility indicates an indirect role for Ca^{2+}, suggesting the possible involvement of some factor(s) lost or perhaps irreversibly inactivated during the wash. Increasing Ca^{2+} on the matrix side of the inner membrane reduces the open probability in what appears to be a manner similar to the effect on the MCC activity (Kinnally *et al.*, 1991). Recently, Klitsch and Siemen (1991) described a block by ADP, ATP, GDP, GTP, and GMP of apparent "whole mitoplast" currents that they attribute to the 107 pS activity. In contrast, no effect was observed on the single-channel activity by others with ADP (Szabó and Zoratti, 1992) and ATP (Inoue *et al.*, 1991). Yet ADP is known to affect MCC activity (Szabó and Zoratti, 1992). This discrepancy remains to be resolved. Klitsch and Siemen (1991) compare a variety of properties in brown adipocyte mitoplasts and conclude that the 107 pS activity and the uncoupling protein are not identical.

Pharmacology of the 107 pS Activity

A number of drugs, many known to affect mitochondrial permeability, inhibit the 107 pS activity (Table II) although many others have been found not to be effective (Sorgato *et al.*, 1989). Each of the cationic amphiphiles tested (Antonenko *et al.*, 1991b) and antimycin A (Campo *et al.*, 1990) reduce the open probability of the 107 pS activity, as illustrated in Fig. 6 by amiodarone. In this case, the closed time increases dramatically while the burst length decreases.

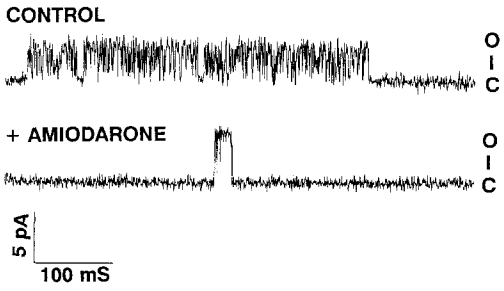

Fig. 6. The effect of amiodarone on 107 pS activity. Current traces show transitions corresponding to 104 pS in the control and 170 pS in the presence of 4 μM amiodarone. Other conditions as in Fig. 1.

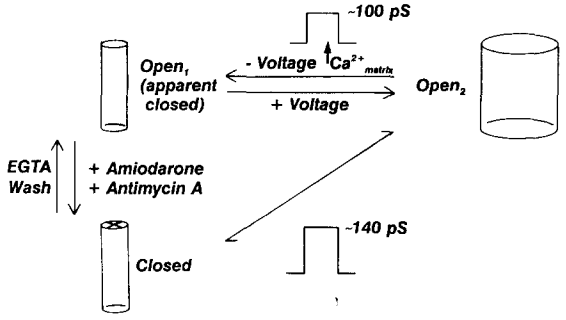

Fig. 7. Model for the 107-pS activity. This voltage-dependent channel is activated by washing mitoplasts with EGTA. Barrel diameter is meant to represent limiting pore diameter. Transitions of 107 or ~140 pS occur to the same open state (open$_2$) from different states, i.e., the fully closed state or the apparent closed state, open$_1$.

The flickering rate and the mean open time do not change significantly when averaged over several patches. In the plasma membrane of cardiomyocytes (Kolhardt and Fichtner, 1988), amiodarone is found to decrease the open probability of Na^+ channels without changing the open time or the unit conductance. In contrast, in our experiments, amiodarone and propranolol increase the transition size by ~40 pS (Antonenko *et al.*, 1991b) (Fig. 6). A decrease in the apparent closed (background) current level is always observed with the addition of amiodarone. There are a number of ways of interpreting these findings. However, the simplest is that amiodarone may affect the apparent closed state (see the state open$_1$ in the model of Fig. 7). Alternatively, amiodarone may have multiple effects. The ~140 pS conductance may represent a substate which is normally obscured by the high occupancy of the 107 pS level and/or it may increase the occupancy of the 140 pS conductance level.

107 pS Activity Model

We propose the 107 pS activity has a minimum of three states as shown in Fig. 7. In agreement with this, the bursting behavior of this activity (Antonenko *et al.*, 1991b) may be attributed to the existence of at least two closed states as it has been in other systems (Moczydlowski, 1986). EGTA washing of mitoplasts causes an activation from the closed state and occupation of the open$_1$ (apparent closed) state. Drugs (e.g., antimycin A) would increase the probability of occupying the closed state. Current transitions corresponding to 107 pS occur between open$_1$ and open$_2$ states and the effects of voltage and Ca^{2+} are as shown. Transitions between the closed and open$_2$ states have a higher unit conductance (~140 pS) than those normally observed. Transitions between the closed and open$_2$ states can occur (e.g., with amiodarone) but are less probable than between the two open states. There is additional evidence for substates. We have observed ~50 pS transitions especially at potentials $\geqslant$ 50 mV which we ascribe to a 107 pS substate, and Klitsch and Siemen (1991) report channel openings of 1/3, 1/2, and 2/3 of the full amplitude associated with 108 pS activity. Also, Klitsch and Siemen (1991) needed three and two exponentials, respectively, to fit the open and closed time distributions, which is also consistent with the existence of multiple substates.

LCC ACTIVITY

A third class (LCC) of activity has been described in native IMM membranes (Antonenko *et al.*, 1991a). LCC displays a low unit conductance (~15 pS) and is activated by alkaline pH (see Table I). It is inhibited by Mg^{2+} and a variety of drugs (see Table II). The IMM reconstitution studies of Moran *et al.* (1990) describe a number of low-conductance transitions which may correspond to different channels. Recently, Inoue *et al.* (1991) using fused mitoplasts described a ~10 pS activity (reported in 100 mM asymmetric salt; corresponds to ~15 pS in 150 mM salt). It is inhibited by ATP, 4-aminopyridine, and glybenclamide. Unlike 107 pS activity and MCC which have weak selectivity, the ~10 pS activity is K^+ selective. Like LCC, this activity is not strongly voltage dependent. We are presently trying to determine if LCC and the ~10 pS activity correspond to the same channel. A pH-dependent inner membrane anion channel (IMAC) was originally postulated from swelling studies done with mitochondiral suspensions by Selwyn *et al.* (1979). It was further characterized by

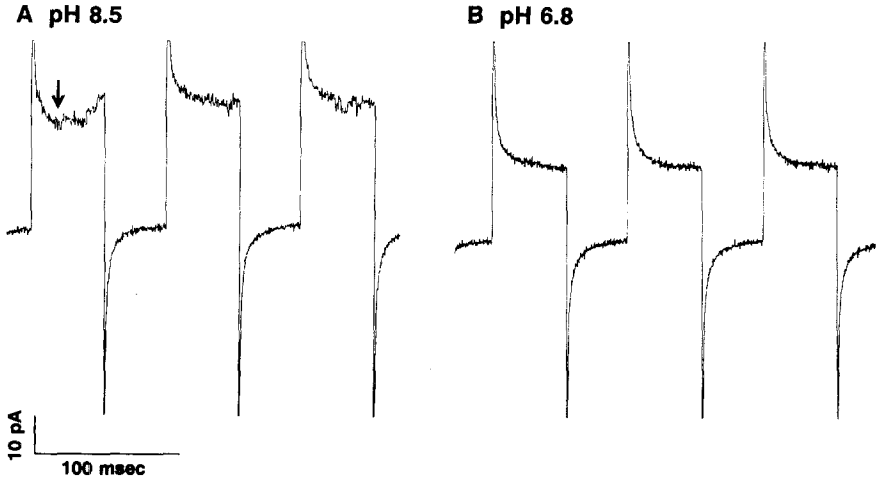

Fig. 8. Single-channel transitions of LCC: current traces from excised IMM patch. The pipette solution was 0.15 M KCl, 5 mM HEPES, and 10 μM $CaCl_2$, pH 6.8 and the bath was changed with perfusion to the same medium at either (A) pH 8.5 or (B) pH 6.8. An oscillating voltage (10 Hz) was applied between 40 (lower) and 190 mV (upper) in order to visualize the single-channel transitions of ~ 15 pS at 190 mV. Arrow indicates opening of three 15 pS channels. An oscillating voltage was used as patch stability was often a problem at the voltages needed to resolve the small transitions. The bandwidth was limited to 2 kHz.

later work (e.g., Garlid and Beavis, 1986), and the pharmacology was explored by Beavis (1989). Much of our data suggests that IMAC and LCC may be the same.

LCC Is Activated by Alkaline pH

When the pH of the matrix side of the IMM membrane in excised patches is changed from 6.8 and 8.3 (by perfusion of the bath), an increase in current is observed (Antonenko *et al.*, 1991a). The current shift to higher levels is completely reversed by perfusion with pH 6.8 media and the system can be cycled repeatedly between pH 6.8 and 8.3. The effect is not voltage dependent as the same conductance changes are seen at ± 40 and ± 60 mV. Gradually changing the pH (by perfusion with intermediate pH) resulted in gradual mean current changes. This is consistent with the presence of several small channels in the patch whose open probability increases with alkaline pH rather than a single larger channel. Associated with the current increase at pH 8.3 is an increase in noise that can be resolved into channel activity by alternating voltages between $+40$ and $+190$ mV as shown in Fig. 8. The data indicate a unit conductance of 15.3 ± 1.9 pS (mean $\pm$ S.D., $n = 17$), which corresponds well to the unit conductance extrapolated from studies done at 0.5 to 2 M KCl (Antonenko *et al.*, 1991a). In Fig. 8, the initial spikes and the gradual decrease in current correspond to capacitance effects. Current transitions indicating the presence of at least three LCC in the patch is seen in the first 190 mV pulse. The ~ 90 pS conductance increase of this patch with pH 8.5 indicates there are probably six LCC active in this patch. A lower current level and a lack of transitions are seen at pH 6.8.

LCC Activity Model

We are proposing a two-state (open and closed) model for LCC as shown in Fig. 9. While alkaline pH increases occupation of the open state, the closed state is favored by low pH, drugs, tributyltin, and Mg^{2+} (Antonenko *et al.*, 1991a). In terms of physiological impact, it is unlikely that LCC are open even if the matrix is alkaline (as it would be during metabolism) as the matrix Mg^{2+} level is greater than that needed

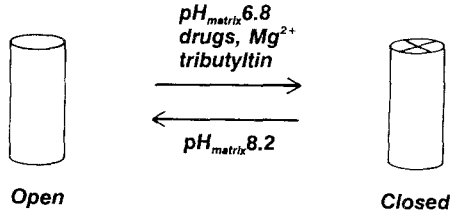

Fig. 9. Model for LCC activity. LCC has a much higher open probability at alkaline pH than at pH 6.8. It is inhibited by Mg^{2+}, tributyltin, amiodarone, propranolol, and quinine.

for current inhibition (0.3 mM for 50% inhibition at pH 8.2).

Are IMAC and LCC the Same Channel?

LCC probably corresponds to the inner membrane anion channel (IMAC) postulated by others (reviewed by Beavis, 1992) for the following reasons: (a) both are activated by alkaline pH; (b) both are inhibited by comparable levels of Mg^{2+} (Beavis and Powers, 1989; Antonenko *et al.*, 1991a); (c) both are inhibited by amiodarone, propranolol, and quinine at about the same concentrations (Beavis, 1989, Antonenko *et al.*, 1991a); (d) both are inhibited by tributyltin (Powers and Beavis, 1991; Antonenko *et al.*, 1991a); (e) Cl^- fluxes estimated for IMAC by Beavis and Garlid (1987) and Beavis (1989) (done photometrically) and Cl^- fluxes calculated from the conductance increase induced by alkaline pH in patch clamp studies at very low potentials are both in the same range, i.e., 200–500 nmoles/min mg protein; (f) the selectivity of IMAC for various anions suggests a small effective pore size, and the unit conductance of LCC is only 15 pS; (g) IMAC is thought to be anion selective, and the patch anion permeability is higher at pH 8.3 than 6.8 (Antonenko, 1991a). Single-channel selectivity experiments with LCC are now in progress. It is not likely that the ~10 pS activity (Inoue *et al.*, 1991) corresponds to IMAC as it is cation rather than anion selective (at pH 7.2), unless selectivity changes with pH.

DISCUSSION

Differentiation of IMM Channels and Their Regulation

Each of the three classes of IMM channels found in high abundance in the IMM differ in character and regulation. Both MCC and 107 pS activities appear to have substates. While the 107 pS activity is strongly voltage dependent, LCC is not between ±60 mV and MCC has a variable voltage dependence. A pH (~6–9) dependence is observed for LCC but not for MCC (unpublished results) or for the 107 pS activities (Sorgato *et al.*, 1987). Pharmacological differences are now being examined (see Table II). Cyclosporine A selectively inhibits MCC but not the 107 pS activity (Szabó and Zoratti, 1991). Amiodarone increases the conductance of the 107 pS activity (Antonenko *et al.*, 1991b). Low concentrations of propranolol inhibit anion permeability with little effect on cation permeability (Antonenko *et al.*, 1991a), which suggests that LCC may be more sensitive to the drug than MCC. Our preliminary results indicate that MCC may be more sensitive to antimycin A than 107 pS activity (not shown). These findings suggest that at least MCC and the 107 pS activities are due to distinct channels. The final determination must await channel isolation and characterization (see Moran and Sorgato, 1992) as these results may still be due to differences in functional states.

We found that each of the channel classes are regulated by divalent cations. This is not surprising considering the pivotal role of Ca^{2+} as a secondary messenger and in biological regulation in general. Several effects are intriguing. The irreversibility of the effect of calcium in the activation of MCC and 107 pS activities in our hands suggest the involvement of other factors such as modulators which are lost or possibly inactivated after excision of the patch from the mitoplast. Modulators are also suggested by the activation of MCC by voltage. Presumably the modulator favors the closed configuration of the channel and is lost at high voltage. Similar activation has also been observed in cell membrane channels (e.g., Blatz and Magleby, 1983 and Gray *et al.*, 1984), and a modulator was proposed. In addition, the outer mitochondrial membrane channel VDAC appears to be closed by a protein modulator (Holden and Colombini, 1988). Differences in results may also find root in the presence or absence of viable modulators. Bertl and Slayman (1991), studying a cation-selective tonoplast channel in yeast, found control by Ca^{2+} at different concentrations after treatment with reducing agents.

Are These Channels Free to Open *in situ*?

Our results on regulation strongly suggest that under most physiological conditions all three channel classes are closed. They therefore present no threat to the low permeability of the IMM.

Do conditions exist which would likely open any of these channel classes *in vivo*? Elevated Ca^{2+} levels are associated with both ischemia and cell death and these may trigger mitochondrial channel opening. Alkalinization of the matrix is associated with oxidative phosphorylation but the pH-dependent LCC likely remains closed because of matrix Mg^{2+} levels. As we learn more about their regulation, the physiological implications and the function of IMM channel activation will be clarified.

What Are the Likely Physiological Roles for These Channels?

The channels require activation and are likely to fulfill special physiological functions. It has been suggested that the transfer of peptides through membranes requires water-filled channels (Singer *et al.*, 1987). Furthermore, contact sites have been implicated in the incorporation of cytoplasmically synthesized mitochondrial proteins (e.g., Schwaiger *et al.*, 1987) and the signal sequence of subunit IV of cytochrome c-oxidase interacts with an outer mitochondrial membrane channel (Henry *et al.*, 1989). These findings are consistent with a mechanism involving a two-channel assembly of the type represented in Fig. 5A for MCC. Channels in tandem in a "gap-junction" arrangement between adjacent mitochondria could also explain the connection between mitochondria in a cluster demonstrated physiologically by Amchenkova *et al.* (1988) and shown morphologically (e.g., Bakeeva *et al.*, 1983).

Discussion of other functions is even more speculative. Beavis (1992) suggests a role of IMAC in mitochondrial volume regulation. Gunter and Pfeiffer (1990) also propose a role of IMM channels in volume regulation particularly during metabolic or osmotic stress. While stretch activation (implicated in osmotic mechanisms) of IMM channels has not been observed, this may be due to isolation procedures.

Tedeschi (1981) has proposed a generalized role of nonspecific channels in cation transport in mitochondria. Presumably, the specificity could be controlled by selectivity filters such as small Ca^{2+}-binding polypeptides in series with the channel (Jeng and Shamoo, 1980, Panfili *et al.*, 1983). Drug-sensitive channel behavior of translocases has been demonstrated. For example, ouabain-sensitive channel activity is observed when the Na^+, K^+ ATPase is incorporated into bilayers (Last *et al.*, 1983). Similarly, mitochondrial proteins implicated in Ca^{2+} (e.g., Mironova *et al.*, 1982) or K^+ (Mironova *et al.*, 1981; Costa *et al.*, 1991) transport exhibit channel behavior in reconstituted systems.

IMM channels may also play a role in thermogenesis. Uncoupling protein from brown fat mitochondria has been reconstituted in liposomes and found to have activity suggestive of a channel (e.g., Jezek and Garlid, 1990). This is consistent with the uncoupling of ADP phosphorylation from electron flow in brown fat mitochondria by this protein.

CONCLUSIONS

Three classes of IMM channels have been characterized. It appears likely that one corresponds to the inner membrane anion channel, IMAC, and that another corresponds to the pore responsible for the Ca^{2+}-induced permeability transition. All three classes appear to be highly regulated by a variety of means so that the channels are likely closed under normal conditions. We should be able to define their physiological role by understanding the activation and inactivation of these channels. Some candidate roles being investigated include protein import, part of ion transport assemblies, volume regulation, thermogenesis, and intermitochondrial communication.

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