The mitochondrial permeability transition (MPT) pore is a calcium-sensitive channel in the mitochondrial inner membrane that plays a crucial role in cell death. Here, we show that cytochrome bc1 regulates the MPT in isolated rat liver mitochondria and in CEM and HL60 cells by two independent pathways. Glutathione depletion activated the MPT via increased production of reactive oxygen species (ROS) generated by cytochrome bc1. The ROS producing mechanism in cytochrome bc1 involves movement of the “Rieske” iron-sulfur protein subunit of the enzyme complex, because inhibition of cytochrome bc1 by pharmacologically blocking iron-sulfur protein movement completely abolished ROS production, MPT activation, and cell death. The classical inhibitor of the MPT, cyclosporine A, had no protective effect against MPT activation. In contrast, the calcium-activated, cyclosporine A-regulated MPT in rat liver mitochondria was also blocked with inhibitors of cytochrome bc1. These results indicate that electron flux through cytochrome bc1 regulates two distinct pathways to the MPT, one unregulated and involving mitochondrial ROS and the other regulated and activated by calcium.

Mitochondria play a vital role in cell fate by their regulation of energy metabolism and their involvement in cell death by apoptosis and necrosis (1–3). Mitochondrial function, including ion transport, biogenesis, and ATP formation, requires an intact mitochondrial transmembrane potential (Δψm), which depends upon the generation of an electrochemical proton gradient (ΔpH) across the mitochondrial inner membrane. The ΔpH is generated by three multisubunit protein complexes localized in the mitochondrial inner membrane including respiratory complex I (NADH:ubiquinone dehydrogenase), complex III (cytochrome bc1), and complex IV (cytochrome c oxidase) (4, 5). A crucial event that occurs in mitochondria when a cell dies is loss of the ΔpH and subsequent collapse of the Δψm (6), which can occur because of opening of high conductance permeability transition (MPT) pores in the mitochondrial inner membrane that allow the nonselective diffusion of solutes (<1500 Da) across the membrane with resulting organelle swelling and membrane rupture (6–9). The MPT is known to be activated by Ca2+ and reactive oxygen species (ROS) and inhibited by the potent immunosuppressive agent cyclosporine A (CsA) (7–10). Although many studies have considered that the MPT is due to the formation of a preformed pore complex between the mitochondrial inner and outer membranes involving the adenine nucleotide translocator (ANT), the voltage-dependent anion channel, cyclophilin D (CyD), and a number of accessory proteins (11–15), an alternative view has been that the MPT is not the result of opening of a preformed pore, but the result of increased membrane permeability caused by oxidative damage to pre-existing membrane proteins including the ANT (8, 9).

The mechanism by which CsA inhibits the MPT has been attributed to its inhibitory effect on the peptidyl-prolyl isomerase activity of CyD, which is believed to be required for the formation of an ANT/CyD protein complex required for MPT activation (14–17). However, this MPT model has been questioned by a report showing that when CyD was overexpressed and targeted to mitochondria, it protected cells from oxidants, indicating that it was inhibiting rather than activating the MPT (18). Although in direct contrast to this Li et al. (19) recently found that overexpression of mitochondrial CyD rendered mitochondria more susceptible to the MPT and cells more sensitive to oxidant-mediated injury. The controversy concerning the role of the ANT and CyD in the MPT is further compounded, first, by the recent finding that the MPT was found to be inducible in mitochondria taken from the livers of mice with genetically inactivated ANT isoforms (20), and second, although CsA blocks the MPT in some cases, it is ineffective at MPT inhibition in others (21, 22). This led He and Lemasters (23, 24) to propose an alternative model of the MPT that would account for some of these inconsistencies (9, 11). Their model envisaged both a “CsA-regulated” and an “unregulated” form of MPT based on a study showing two possible conductance modes for the MPT. One mode was activated by Ca2+ and inhibited by CsA, and the other was unregulated.

The dualistic model of He and Lemasters (23, 24) thus reconciles two apparently divergent ideas on the MPT where both the ANT and CyD play an important regulatory role. However, other investigators have considered models of MPT that may involve the ANT and CyD but feature mitochondrial respiratory components other than the ANT as crucial MPT regulators; for example, the work of Fontaine and co-workers (25–27) clearly showed that the MPT is regulated by electron flux through the NADH-ubiquinone-dehydrogenase and that vari-
ous classes of quinone analogs were important MPT regulatory molecules. Whereas we have previously shown that mitochondrial ROS activates the MPT in vivo after GSH depletion and proposed the redox target(s) include the ANT (28). These varied and somewhat controversial reports on the molecular composition and regulation of the MPT indicate that definitive knowledge on this phenomenon is lacking and suggest that concerted efforts should be made to advance our understanding of this crucial mitochondrial event associated with cell death.

In this study we show that cytochrome $bc_1$ is a key regulatory component of the MPT in rat liver mitochondria (RLM) and in leukemic CEM and HL60 cells. Our results indicate a fundamental role for ISP subunit movement in ROS-mediated MPT activation in cells, whereas investigations with RLM indicate that cytochrome $bc_1$ may possess a MPT channel-like function, suggesting that cytochrome $bc_1$ is involved in two distinct pathways to the MPT.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the chemicals were reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO), tetramethylrhodamine methylester (TMRM) and dichlorodihydrofluorescein diacetate (DCFDA) was obtained from Molecular Probes (Eugene, OR). Bongkrekic acid (BkG) was obtained from Calbiochem (La Jolla, CA). Stigmatellin was obtained from Fluka Biochimika, and 2-methoxy antimycin A$_1$ (M), myxothiazol (5 $\mu$m), and myxothiazol-6-methyl-6-decyl-1,4-benzoquinone (deuclyubiquinol) as substrate and 50 $\mu$m cytochrome c. Declyubiquinol was synthesized in the laboratory from decylubiquinone by reduction with sodium borohydride (NaBH$_4$) (31). The reduction of cytochrome c was monitored in a spectrophotometer at 550 versus 530 nm in dual wavelength mode. The data are expressed as percentages of control activity and were determined from five individual isolations that were assayed in triplicate.

**Statistical Analysis**—Statistical analyses were performed using Student’s $t$ test for unpaired data, and $p$ values <0.05 were considered significant. The data are presented as means $\pm$ S.E.

**RESULTS**

GSH Depletion Mediates ROS Increase in CEM and HL60 Cells That Is Decreased by Inhibition of Cytochrome $bc_1$—Fig. 1A shows representative ROS production, and Fig. 1B shows mean ROS production, respectively, in CEM cells after treatment with 5 mM DEM with or without the respiratory complex inhibitors. Increased DCF fluorescence, a measure of ROS production (29, 32), was determined by a shift in DCF fluorescence to the right after DEM treatment (Fig. 1A, panel 1). The figures show that DEM treatment causes a time-dependent increase in ROS production. GSH levels were determined on aliquots of CEM cells treated with 5 mM DEM. Fig. 1B (inset) shows that a time-dependent loss of GSH occurs in CEM cells after cells were treated with 5 mM DEM. Approximately 90% of GSH is lost after DEM treatment for 30 min, at which time ROS increase occurs (Fig. 1B).

The Distal Ubiquinol Oxidation Site of Cytochrome $bc_1$ Is the Predominant Site of ROS Production after GSH Depletion—To determine the major mitochondrial site of ROS production after GSH depletion in CEM cells, we pharmacologically inhibited respiratory complex I (NADH ubiquinone-dehydrogenase), respiratory complex II (succinate dehydrogenase (SDH)), and respiratory complex III (cytochrome $bc_1$) because these respiratory sites are well known to be involved in mitochondrial ROS production (33-36). The cells were co-incubated with DEM with either rotenone (5 $\mu$m), thenoyltrifluoroacetone (TFFA) (5 $\mu$m), stigmatellin (5 $\mu$m), myxothiazol (5 $\mu$m), antimycin A, and 2-MeAA (control for antimycin A, which does not inhibit respiration) (40). Rotenone selectively blocks NADH ubiquinone-dehydrogenase (37), TFFA blocks SDH (38), stigmatellin and myxothiazol block the ubiquinol oxidation (Qo) site of cytochrome $bc_1$ at the distal and proximal niches, respectively (39), and antimycin A blocks the ubiquinol reduction Q$_1$ site of cytochrome $bc_1$ (33, 34). Myxothiazol, which binds to the proximal niche of the Qo ubiquinol oxidation site of cytochrome $bc_1$, and antimycin A, which binds to the Q$_1$ site, inhibited ROS formation (Fig. 1A, panels 2 and 4), whereas stigmatellin, which binds to the distal niche of the Qo site, completely blocked DEM-mediated ROS increase (Fig. 1A, panel 5). The experiment with stigmatellin was extended up to 90 min, but we did not observe any increase in ROS production (data not shown). To confirm that antimycin A inhibited ROS production, we used the structural analog 2-MeAA as a control (this compound does not inhibit respiration) (40). 2-MeAA did not inhibit ROS production in DEM-treated CEM cells compared with antimycin A (Fig. 1A, panel 5). Rotenone and TFFA did not significantly alter ROS increase compared with DEM treatment alone (Fig. 1A, panels 6 and 7). These results indicate that the Qo distal site of cytochrome $bc_1$ is a key source of ROS production because its inhibition abolished ROS production. However, because inhibition of the proximal Qo and the Q$_1$ sites of cytochrome $bc_1$ also reduced ROS production, this further impli-
cates the cytochrome bc₁ as a key ROS-generating site in our experimental setting. An identical set of experiments was performed using HL60 cells with similar results (data not shown).

Cytochrome bc₁ Activity Required for ROS Production after GSH Depletion—The role of cytochrome bc₁ in ROS production in CEM cells was confirmed by determining cytochrome bc₁ activity.
activity in isolated membrane fractions from CEM cells incubated with DEM, DEM + stigmatellin, DEM + myxothiazol, DEM + rotenone, DEM + TTFA, DEM + antimycin A, and DEM + 2-MeAA. Fig. 1C shows means ± S.E. percentage activity of cytochrome bc₁ in CEM cells treated with DEM with or without respiratory inhibitors. The results show that cytochrome bc₁ enzyme activity was not significantly different in CEM cells treated with DEM with or without rotenone, TTFA, or 2-MeAA compared with controls, whereas the compounds stigmatellin, myxothiazol, and antimycin A inhibited cytochrome bc₁ enzyme activity −95%.

**ROS Production by Cytochrome bc₁ Mediates Cell Death by Activation of the MPT—Δψm was determined in CEM cells, after treatment with DEM (5 mM) to deplete GSH, by monitoring the fluorescence of the cationic potentiometric dye TMRM. Fig. 2A shows representative TMRM flow cytometric histograms of the CEM cell population monitored every 30 min for 150 min with or without the inhibitors used in the ROS experiments. Loss of Δψm is indicated by a shift left of the cell population on the x axis of the histogram (log scale). Fig. 2B shows the percentage (mean ± S.E.) of CEM cells with intact Δψm after treatment with DEM with or without inhibitors. Fig. 2A panel 2 shows that the cytochrome bc₁ inhibitors stigmatellin (5 μM) (panel 3) and antimycin A (5 μM) (panel 4) and the ANT inhibitor BgK (50 μM) (panel 8) blocked the loss of Δψm after GSH depletion (Fig. 2, A and B), whereas rotenone (panel 7), TTFA (panel 6), and the antimycin A analog 2-MEAA (panel 5) did not prevent the loss of Δψm. Surprisingly, myxothiazol (Fig. 2, A, panel 2, and B, top panel), which reduced ROS production, did not prevent DEM-mediated loss of Δψm; paradoxically, this inhibitor increased the rate of loss of Δψm compared with DEM treatment alone. The classical MPT inhibitor CyA did not inhibit the loss of Δψm induced by GSH depletion, even at concentrations up to 10 μM (data not shown).

**Inhibition of Cytochrome bc₁ with Stigmatellin or Antimycin A Protects Mitochondrial Ultrastructure in Response to GSH Depletion**—To confirm the protective effect of stigmatellin and antimycin A on mitochondrial integrity, electron microscopy (EM) was performed to visualize mitochondrial ultrastructure after GSH depletion. Fig. 2C shows representative electron micrographs of mitochondrial ultrastructure in control cells (top left panel), cells treated with DEM for 150 min (top right panel), cells treated with DEM for 150 min with 5 μM stigmatellin (bottom left panel), and cells treated with DEM for 150 min with 5 μM antimycin A (bottom right panel). The figure indicates that stigmatellin and antimycin A preserved mitochondrial ultrastructure structure compared with DEM treatment alone. Structural and functional studies were also performed using the HL60 B cell line to determine whether the results observed with CEM cells were a general or cell-specific phenomenon. HL60 cells were treated with DEM (5 mM), and EM and Δψm values were determined every 30 min for a total of 150 min. The results show a time-dependent loss of Δψm that corresponds with significant ultrastructural changes in mitochondria. At 150 min, mitochondrial ultrastructure in HL60 cells was similar to that observed in CEM cells, including increased electron opacity of mitochondrial inner membrane and cristae. These structural changes were prevented by co-incubation of cells with DEM and stigmatellin or antimycin A but not with 2-MeAA (Fig. 3C).

**Cytochrome bc₁ Inhibition Is Required for Protection against ROS-mediated Cell Death**—Because inhibition of cytochrome bc₁ prevented loss of Δψm, we determined cell viability after incubation of CEM cells with 5 mM DEM with or without 5 μM of cytochrome bc₁ inhibitors (stigmatellin, antimycin A and myxothiazol) or cyclosporin A (1–10 μM). Cell viability was performed every 30 min for 150 min using trypan blue exclusion. Cells treated with either DEM alone or DEM with rotenone, TTFA, or 2-MeAA lost cell viability over a similar time, whereas BgK prevented the loss of cell viability (Fig. 4A). Of the cytochrome bc₁ inhibitors, stigmatellin blocked the loss of cell viability induced by DEM, and antimycin A reduced the rate at which cells died, whereas myxothiazol did not prevent the loss of cell viability (Fig. 4B). These results indicate that the inhibition of ROS formation by stigmatellin or antimycin A protects against redox-dependent cell death. BgK, also prevented the loss of cell viability; however, myxothiazol, which reduced ROS production but did not preserve Δψm after GSH depletion, did not prevent the loss of cell viability. CyA, even over a range of concentrations (1–20 μM), did not prevent the loss of cell viability after GSH depletion (data not shown). Cell death was not inhibited by broad spectrum caspase inhibitor benzylxoycarbonyl-VAD-fluoromethyl ketone or the caspase 3 inhibitor DEVD-CHO, suggesting that the predominant death pathway was by necrosis (data not shown).

**Inhibition of Cytochrome bc₁ Blocks the Ca²⁺-activated MPT in RLM**—Because inhibition of cytochrome bc₁ prevented the redox-activated MPT in cells in situ, we determined whether inhibition of cytochrome bc₁ would also prevent the MPT induced by Ca²⁺ in RLM. Fig. 5A shows a representative example of the effect of different respiratory complex inhibitors on the Ca²⁺-activated MPT in RLM. Stigmatellin (1 μM, trace a) and rotenone (1 μM, trace b) completely prevented mitochondrial swelling induced by 100 μM Ca²⁺ with similar potency as CsA (1 μM, trace c). TTFA did not prevent mitochondrial swelling (trace d). Fig. 5B shows a representative example of the effects of antimycin A (1 and 10 μM, traces a and c, respectively) and myxothiazol (1 μM, trace b) on the Ca²⁺-activated MPT in RLM. Fig. 5B (trace d) shows the effect of 2-MeAA on Ca²⁺-induced swelling of RLM; this trace also represents the profile of RLM swelling induced by Ca²⁺ alone. These results indicate that electron flux through cytochrome bc₁ and the NADH:ubiquinone dehydrogenase regulates the Ca²⁺-activated MPT in RLM.

**DISCUSSION**

In this study we show that mammalian cytochrome bc₁ is a key regulator of the MPT in CEM and HL60 cells and RLM. In cells, mitochondrial ROS generated during ISP movement at the Qo site of cytochrome bc₁ activates the MPT, which is insensitive to CsA and results in necrosis. In contrast, in RLM the MPT is activated by Ca²⁺ load and is blocked by inhibitors of the Qo site of cytochrome bc₁ but is sensitive to CsA. These results indicate that cytochrome bc₁ plays a key role in regulation of the regulated MPT in RLM and the unregulated MPT in cells.

DEM was used to deplete cellular GSH and induce redox stress in CEM and HL60 cells as previously shown (28, 29). The increase in mitochondrial ROS in CEM cells was blocked by inhibition of cytochrome bc₁ but not NADH:ubiquinone dehydrogenase or SDH (Fig. 1B). Because these respiratory complexes have each been associated with ROS production (34), our results indicate that under these experimental conditions, cytochrome bc₁ is the principal mitochondrial site of ROS formation as previously found (28, 29). Inhibition of cytochrome bc₁ at either the Qo or Qi site of cytochrome bc₁ inhibited ROS production (measured by the relative DCF fluorescence of cells); however, the distal Qi niche inhibitor antimycin completely blocked ROS production, whereas myxothiazol inhibited ROS production (Fig. 1B, bottom panel). These results indicate that mitochondrial ROS are produced principally at the Qo site of cytochrome.
FIG. 2. A, representative TMRM flow cytometric histograms of CEM cells treated with DEM (5 mM) alone (panel 1), DEM (5 mM) with myxothiazol (5 μM) (panel 2), stigmatellin (5 μM) (panel 3), antimycin A (5 μM) (panel 4), 2-MeAA (5 μM) (panel 5), TTFA (5 μM) (panel 6), rotenone (5 μM) (panel 7), and BgK (50 μM) (panel 8) for 0, 30, 60, 90, 120, and 150 min, washed in PBS, and suspended in PBS containing 10 mM glucose. The cells were loaded with TMRM (250 nM) for 15 min, and the red fluorescence was immediately measured by flow cytometry using the FL-3 setting as described under “Experimental Procedures.” The figure shows a representative example of three independent experiments. In each analysis, 10,000 events were recorded. B, top panel, percentage of CEM cells with an intact ΔΨm determined by relative TMRM fluorescence intensity. The cells were treated with DEM (5 mM) with or without myxothiazol (5 μM) (panel 2), stigmatellin (5 μM) (panel 3), antimycin A (panel 4), and 2-MeAA (5 μM) for 0, 30, 60, 90, 120, and 150 min, washed in PBS, and suspended in PBS containing 10 mM glucose. The cells were loaded with TMRM (250 nM) for 15 min, and the red fluorescence was immediately measured by flow cytometry using the FL-3 setting as described under “Experimental Procedures.” The data are expressed as the means ± S.E. (n = 3). In each analysis, 10,000 events were recorded. Bottom panel, percentage of CEM cells with an intact ΔΨm determined by relative TMRM fluorescence intensity. The cells were treated with DEM (5 mM) with or without TTFA (5 μM), rotenone (5 μM), and BgK (50 μM) for 0, 30, 60, 90, 120, and 150 min, washed in PBS, and suspended in PBS containing 10 mM glucose. The cells were loaded with TMRM (250 nM) for 15 min, and the red fluorescence was immediately measured by flow cytometry using the FL-2 setting as described under “Experimental Procedures.” The data are expressed as the means ± S.E. (n = 3). In each analysis, 10,000 events were recorded. C, the figure shows the effects of DEM treatment on CEM mitochondrial ultrastructure. Electron microscopy (TEM) was performed as described under “Experimental Procedures.” The figure shows the effects of untreated cells (top left panel), DEM-treated cells (top right panel), DEM with stigmatellin (5 μM) (bottom left panel), and antimycin A (bottom right panel).
The difference in the Qo site-binding mechanism of the inhibitors myxothiazol and stigmatellin may be important in elucidating the mechanism involved in ROS formation at the Qo site, because these two inhibitors have dramatically different effects on the mobility of the extramembrane domain of the ISP (41–43). Stigmatellin immobilizes the ISP domain on the surface of cytochrome b inhibiting the Q cycle, whereas myxothiazol allows movement of the ISP but inhibits the enzyme by competitive inhibition at the ubiquinone oxidation site (44). These observations suggest that ROS formation by cytochrome bc₁ involves the mobility of the ISP as a key feature. Although the generation of superoxide by cytochrome bc₁ is not new, the concept that ISP movement is mechanistically linked to ROS during the Q cycle is noteworthy because, to our knowledge,
this is the first report to propose the ISP as directly involved in ROS production. In support of this novel idea, superoxide production, whereas myxothiazol only partially prevented superoxide formation (39). A report by Sun and Trumpower (45) using bovine heart and S. cerevisiae cytochrome bc$_1$ complexes also showed that stigmatellin eliminated superoxide formation compared with myxothiazol and antimycin A.

We next considered that if the mechanism of ROS formation involved ISP mobility, inhibition of ISP movement should not only prevent ROS formation but also prevent the toxicity from uncontrolled ROS production. Cell viability experiments clearly showed that ROS inhibition of ISP mobility with stigmatellin preserved cell viability compared with cells treated with DEM alone (Fig. 4A). The Qi site inhibitor antimycin A also preserved cell viability, although to a lesser extent than stigmatellin; however, surprisingly myxothiazol, which reduced ROS formation, did not inhibit cell death (Fig. 4A). To investigate this we first confirmed the efficacy of the respiratory complex inhibitors on cytochrome bc$_1$ enzyme activity using membrane fractions of CEM cells treated with DEM with or without inhibitors. The results showed that cytochrome bc$_1$ activity was almost completely (~95%) inhibited in cells treated with DEM with or without either stigmatellin, myxothiazol, or antimycin A (Fig. 1C). We next considered that myxothiazol could be intrinsically toxic at the concentration used in the experiment. To test this, we treated cells with a combination of DEM and stigmatellin with myxothiazol expecting that if myxothiazol were intrinsically toxic, inhibition of ROS production by stigmatellin would fail to rescue cells. We found that cells were rescued from loss of viability induced by DEM with myxothiazol by co-incubation with stigmatellin, indicating that if myxothiazol were intrinsically toxic, inhibition of ROS formation by stigmatellin would fail to rescue cells. We found that cells were rescued from loss of viability induced by DEM with myxothiazol by co-incubation with stigmatellin, indicating that if myxothiazol were intrinsically toxic, inhibition of ROS formation by stigmatellin would fail to rescue cells. We found that cells were rescued from loss of viability induced by DEM with myxothiazol by co-incubation with stigmatellin, indicating that if myxothiazol were intrinsically toxic, inhibition of ROS formation by stigmatellin would fail to rescue cells. We found that cells were rescued from loss of viability induced by DEM with myxothiazol by co-incubation with stigmatellin, indicating that if myxothiazol were intrinsically toxic, inhibition of ROS formation by stigmatellin would fail to rescue cells. 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ROS will continue to be released into the mitochondrial matrix, resulting in increased intramitochondrial oxidation (Scheme 1). Moreover, because myxothiazol is not specific for cytochrome bc, but also inhibits the NADH:ubiquinone dehydrogenase and increases superoxide formation in the mitochondrial matrix side, significantly increased mitochondrial protein oxidation might be expected using this inhibitor (48–50). There is some evidence for this proposition, because ∆ψm of CEM cells was lost at a significantly earlier time after treatment with DEM with myxothiazol compared with DEM alone (Fig. 3, B and C). The decreased level of ROS detection in CEM cells treated with DEM with myxothiazol compared with DEM alone (Fig. 3, panel 2) (51). These results therefore indicate that cytochrome bc is crucial in the redox-activated MPT and that increased ROS production in the mitochondrial matrix is likely to be the major cell compartment where ROS are produced and are responsible for the loss of ∆ψm and the MPT.

If ROS production in the matrix and subsequent oxidative damage to matrix proteins were involved in the redox-MPT, we would expect that cytochrome bc inhibition would preserve mitochondrial ultrastructure and ∆ψm, which we determined in two independent cell lines including CEM and HL60 cells. The results clearly showed that GSH depletion caused loss of ∆ψm together with significant mitochondrial ultrastructural changes in mitochondria compared with mitochondria in control cells (Figs. 2A and 3A). These GSH-dependent changes included a characteristic increased electron density of inner mitochondrial membranes and cristae in both HL60 and CEM cells (Figs. 2C and 3A). The ultrastructural changes resulting from GSH depletion were not observed in mitochondria of cells co-incubated with DEM and stigmatellin or antimycin A (Figs. 2C and 3, B and C) but were apparent in mitochondria of cells treated with the antimycin A analog 2-MeAA (Fig. 3C). The ANT has been previously implicated as a key protein target in the redox-MPT, because the BgK, which inhibits the ANT at the matrix side, also blocked loss of ∆ψm and cell death (Fig. 3B). Our results suggest that ANT is a key protein involved in the redox-MPT and that the ATP/ADP-binding site of the ANT, which is excluded by binding the ligand BgK, is a key MPT oxidative target protein (52). The redox-MPT is characterized as unregulated because CsA, which was used over a wide range of concentrations (1–20 μM), did not inhibit ROS production or prevent the loss of ∆ψm and cell death (data not shown) (23, 24).

Our results suggest that cytochrome bc-dependent ROS production activates the MPT described by the model proposed by Kowaltowski et al. (8) in 2001 (8) and recapitulates the unregulated MPT model proposed by He and Lemasters (23, 24).

Because cytochrome bc was clearly involved in the unregulated MPT by its ROS producing activity, we next determined whether this respiratory complex was a regulator of the Ca2+-dependent MPT. For these studies we isolated RLM by standard procedures and performed the classical mitochondrial swelling test as an indicator of MPT activation in response to either increased Ca2+ load. Stigmatellin prevented large amplitude swelling of RLM induced by 100 μM Ca2+ with similar (equimolar) efficacy as CsA. Myxothiazol and antimycin A also inhibited swelling of RLM but to a lesser extent (Fig. 5). 2-MeAA was used as a control for antimycin A and did not prevent Ca2+-induced large amplitude swelling in RLM compared with antimycin A (Fig. 3C). Taken together, our results indicate that two distinct pathways to the MPT exist, as previously suggested (8, 9, 11–15, 23, 24), and that cytochrome bc may be a key regulator factor in both pathways.

Although the proteins composing and regulating the MPT are still unknown, previous reports clearly indicate that the MPT is regulated by electron flux through the NADH:ubiquinone dehydrogenase in cells as well as isolated mitochondria (25–27). Our study, using inhibitors of 1) the Qo ubiquinol catalytic site, 2) the Qi site, and 3) ISP movement show that

**SCHEME 1.** Topology of mitochondrial superoxide production in the presence of antimycin A and myxothiazol. This scheme was adapted from the work of Trumpower (46) and Boveris and Cadenas (47).

**Fig. 6.** A model of cytochrome bc involvement in regulated and unregulated MPT. The model depicts the unregulated MPT activated by mitochondrial ROS generated by cytochrome bc in the mitochondrial matrix and the regulated MPT activated by electron flux through cytochrome bc. In the model we show the idea that a respiratory supercomplex formed by NADH:ubiquinone dehydrogenase and cytochrome bc is involved in regulation of the MPT.
cytochrome bc1 is a key regulatory component of the MPT by its ability to generate ROS and its potential to activate the MPT in response to increased Ca\(^{2+}\). Metabolic flux control theory shows that the NADH:ubiquinone dehydrogenase and cytochrome bc1 may be associated as a single enzyme with coenzyme Q as a common substrate (53, 54). Schagger (54) has proposed that these two respiratory components form a stable core respirasome in humans, and similar respiratory complex associations have been found in plant mitochondria (55). Based on these notions, our work, and the investigations of others, we propose a novel MPT model regulated by a respiratory supercomplex formed by NADH:ubiquinone dehydrogenase and cytochrome bc1 as well as the ANT (Fig. 6). The role of quinones in this model is implicated from the work of Fontaine and co-workers (26, 27) as well as from our previous work (29).

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