Inhibition of Complex I by Ca^{2+} Reduces Electron Transport Activity and the Rate of Superoxide Anion Production in Cardiac Submitochondrial Particles†

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ABSTRACT: Declines in the rate of mitochondrial electron transport and subsequent increases in the half-life of reduced components of the electron transport chain can stimulate $O_2^{\bullet-}$ formation. We have previously shown that, in solubilized cardiac mitochondria, Ca^{2+} mediates reversible free radical-induced inhibition of complex I. In the study presented here, submitochondrial particles prepared from rat heart were utilized to determine the effects of Ca^{2+} on specific components of the respiratory chain and on the rates of electron transport and $O_2^{\bullet-}$ production. The results indicate that complex I is inactivated when submitochondrial particles are treated with Ca^{2+}. Inactivation was specific to complex I with no alterations in the activities of other electron transport chain complexes. Complex I inactivation by Ca^{2+} resulted in the reduction of NADH-supported electron transport activity. In contrast to the majority of electron transport chain inhibitors, Ca^{2+} suppressed the rate of $O_2^{\bullet-}$ production. In addition, while inhibition of complex III stimulated $O_2^{\bullet-}$ production, Ca^{2+} reduced the relative rate of $O_2^{\bullet-}$ production, consistent with the magnitude of complex I inhibition. Evidence indicates that complex I is the primary source of $O_2^{\bullet-}$ released from this preparation of submitochondrial particles. Ca^{2+} therefore inhibits electron transport upstream of site-(s) of free radical production. This may represent a means of limiting $O_2^{\bullet-}$ production by a compromised electron transport chain.

Mitochondria have long been recognized for their role in the maintenance of cellular energy status and function. Mitochondria are also capable of accumulating Ca^{2+} (*1*−*5*) and producing oxygen-derived free radicals (*6*−*13*). Recent interest in these organelles has been stimulated by their acknowledged role in necrotic and apoptotic cell death. Both forms of cell death are dependent on dramatic changes in mitochondrial function and/or structure and can be initiated by disruption of Ca^{2+} and/or redox homeostasis (*14*−*22*). Mitochondria are therefore responsive to alterations in Ca^{2+} status, produce highly reactive pro-oxidants, and integrate a variety of cellular alterations that ultimately determine the fate of the cell. Important areas of investigation seek to define how these processes are regulated and to identify molecular determinants that shift the balance from normal physiological control to pathophysiological disintegration.

An increase in the mitochondrial Ca^{2+} concentration can activate the matrix dehydrogenases pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and NAD^+-isocitrate dehydrogenase (*23*−*31*). These stimulatory effects are postulated to couple the energetic demands of muscle contraction and relaxation with ATP synthesis (*32*). Unabated, mitochondrial Ca^{2+} uptake can, however, induce large-amplitude swelling, disruption of mitochondrial integrity and function, cytochrome *c* release, and cellular necrosis and/or apoptosis (*4*, *15*, *17*−*22*). Similarly, free radicals are capable of reversibly altering mitochondrial function. Redox-dependent inhibition of α-ketoglutarate dehydrogenase (*33*−*35*) may underlie an antioxidant response limiting reducing equivalents required for the production of superoxide anion and/or protecting key sulfhydryl residues from irreversible oxidative modification (*36*−*38*). Oxygen-derived free radical species can, however, induce irreversible inactivation of protein function (*39*, *40*) and irreparable loss of mitochondrial respiratory activity and ATP synthesis (*41*−*44*). Evidence indicates that mitochondrial Ca^{2+} uptake and free radical production are interrelated (*45*).

Ca^{2+} can induce declines in the rate of electron transport through the disruption of the inner mitochondrial membrane (*17*−*20*, *22*, *46*, *47*). Declines in the rate of electron transport and increases in the half-life of reduced components of the electron transport chain can increase the rate of $O_2^{\bullet-}$ formation (*6*−*13*). Conversely, a decrease in the rate of electron transport may diminish the proton gradient and subsequently the rate of uptake of Ca^{2+} by the mitochondria (*1*−*5*). Reports on the effects of Ca^{2+} on mitochondrial pro-oxidant production have varied (*2*, *20*, *48*, *49*). This is likely due to differences in the experimental model that is utilized, the method of free radical detection, and the manner in which Ca^{2+} is administered (*20*, *45*). Our study sought to determine mechanisms by which Ca^{2+} alters mitochondrial electron transport and the effects of these alterations on $O_2^{\bullet-}$ production. Submitochondrial particles isolated from cardiac

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tissue were utilized to distinguish between the contribution(s) of distinct events. We present evidence that Ca^{2+} specifically inhibits complex I, resulting in a reduction in both electron transport chain activity and the rate of $O_2^{\bullet-}$ production. The delicate balance that exists between these processes is discussed.

MATERIALS AND METHODS

Reagents and Animals

Antimycin A, myxothiazol, ubiquinone-1, and superoxide dismutase (CuZnSOD)[1] were purchased from Sigma. Hydroethidine was purchased from Molecular Probes. Male Sprague-Dawley rats (250−300 g) were obtained from Harlan Laboratories.

Isolation of Submitochondrial Particles

Male Sprague-Dawley rats were anesthesized by intramuscular administration of a mixture of xylazine, ketamine·HCl, and acepromazine (3:3:1) (0.5−0.75 mL/kg). Following midline thoracotomy and pericardiectomy, hearts were excised and perfused with 10 mL of ice-cold isolation buffer [210 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, and 10 mM MOPS (pH 7.4)] to remove blood. Hearts were then frozen in liquid N_2 and pulverized. Pulverized tissue was placed into 20 mL of 10 mM MOPS and 1.0 mM EDTA (pH 7.4) and homogenized by 4 × 4 s passes using a polytron homogenizer followed by 15 passes with a Potter-Elvejem homogenizer. Homogenate was then centrifuged at 750*g* for 5.0 min, and the supernatant was collected. The supernatant was centrifuged at 10000*g* for 20 min and the pellet resuspended in 12 mL of 10 mM MOPS and 1.0 mM EDTA (pH 7.4) (2.0−5.0 mg/mL). Following two cycles of freezing (liquid N_2) and thawing, the solution was centrifuged at 10000*g* for 20 min. The pellet was then resuspended in 6.0 mL of 10 mM MOPS and 1.0 mM EDTA (pH 7.4) followed by sonication on ice (8 × 15 s with 30 s intervals, sonic dismembrator output of ∼20 W). The sonicated preparation was then centrifuged at 10000*g* for 7.0 min to remove unbroken mitoplasts. The supernatant was centrifuged at 40000*g* for 60 min. The resulting pellet, representing submitochondrial particles, was resuspended in 10 mM MOPS (pH 7.4) at a protein concentration of 1.0 mg/mL. As detailed in Results, submitochondrial particles prepared as described were inside-out vesicles devoid of the antioxidant enzyme MnSOD. While ATP synthase activity was retained, all experiments were performed under nonenergized conditions (no added Mg^{2+}/ATP). Unlike certain preparations (*50*), submitochondrial particles were prepared and assayed under conditions of low osmotic strength to ensure removal of antioxidant enzymes and analysis of electron transport chain function under maximal rate conditions. Thus, this preparation represented uncoupled submitochondrial particles as evidenced by the observation that the uncoupler, 2,4-dinitrophenol, exerted no effect on the maximal rates of NADH and succinate oxidase activity.

Ca^{2+} Incubations and Enzymatic Analyses

Submitochondrial particles (25 μg/mL) were incubated with varying concentrations of Ca^{2+} for 20 min at 37 °C in 10 mM MOPS (pH 7.4) followed by equilibration at 25 °C for 5.0 min. All assays were then performed in 50 mM KCl and 10 mM MOPS (pH 7.4, 25 °C) with 25 μg/mL submitochondrial particles except where noted.

NADH Oxidase Activity. NADH-supported electron transport was assessed spectrophotometrically as the rate of NADH oxidation (340 nm, $\epsilon = 6200\ M^{-1}\ cm^{-1}$) following addition of 200 μM NADH.

Succinate Oxidase Activity. Succinate-supported electron transport was assessed as the rate of O_2 consumption upon addition of 10 mM succinate using a Clark-style electrode.

Complex I. Following addition of 50 nM antimycin A and 100 μM ubiquinone-1, complex I activity was initiated with NADH (100 μM). Activity was measured spectrophotometrically as the rate of NADH oxidation (340 nm, $\epsilon = 6200\ M^{-1}\ cm^{-1}$). Activity required the presence of ubiquinone-1 and was inhibited by rotenone, indicating that NADH utilization was dependent on complex I activity.

Complex II. Following addition of 50 nM antimycin A and 100 μM ubiquinone-1, complex II activity was initiated with succinate (10 mM). Activity was measured spectrophotometrically as the rate of reduction of ubiquinone-1 to ubiquinol-1 (280 nm, $\epsilon = 13\,700\ M^{-1}\ cm^{-1}$). Activity required the presence of ubiquinone-1 and was inhibited by malonate, indicating that succinate utilization and ubiquinone-1 reduction were dependent on complex II activity.

Complex III to IV. The flow of electrons from complex III through complex IV was initiated upon addition of ubiquinol-1 (100 μM). Activity was measured spectrophotometrically as the rate of oxidation of ubiquinol-1 to ubiquinone-1 (280 nm, $\epsilon = 13\,700\ M^{-1}\ cm^{-1}$). Antimycin A or CN^- inhibited activity, indicating transport of electrons from complex III through complex IV.

Superoxide Anion. The oxidation of hydroethidine by $O_2^{\bullet-}$ to the recently described fluorescent product 2-hydroxyethidium (*51*, *52*) was assessed. Briefly, fluorescent signals were recorded (excitation at 480 nm and emission at 567 nm) over time utilizing 5.0 μM hydroethidine and 500 μM NADH or 10 mM succinate. CuZnSOD (8.0 units/mL) was used to test the specificity of the measurement for superoxide anion. Where indicated, antimycin A (50 nM) or myxothiazol (50 nM) was included. It should be noted that oxidation of hydroethidine by $O_2^{\bullet-}$ produced a compound with properties (reverse phase HPLC elution profile and fluorescence properties) consistent with 2-hydroxyethidium and not ethidium as recently reported (*51*, *52*). Nevertheless, the structurally related compound ethidium was used as a standard to estimate the relative rate of $O_2^{\bullet-}$ production due to the absence of commercially available 2-hydroxyethidium.

RESULTS

Characterization of Submitochondrial Particles. Electron transport chain activity and superoxide anion generation by intact mitochondria reflect the rates of (1) NADH production, (2) NADH utilization, (3) production of $O_2^{\bullet-}$ from various sources, and (4) $O_2^{\bullet-}$ removal. Each of these processes may be influenced by Ca^{2+}, thus complicating the interpretation of results. The use of submitochondrial particles supplied with known concentrations of NADH and devoid of antioxidant enzymes allows for the direct assessment of the contribution of specific electron transport chain alterations

[1] Abbreviations: SOD, superoxide dismutase; SMP, submitochondrial particle.

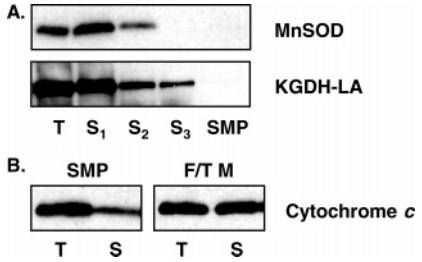

FIGURE 1: Characterization of cardiac submitochondrial particles. (A) During the isolation of rat heart submitochondrial particles, the relative level of MnSOD and KGDH-lipoic acid (LA) in each fraction [total lysate (T), supernatant from the mitoplast pellet (S_1), supernatant following freezing and thawing of mitoplasts (S_2), supernatant from the submitochondrial particle pellet (S_3), and submitochondrial particles (SMP)] was assessed by Western blot analysis using anti-MnSOD and lipoic acid antibodies, respectively. Volumes for each fraction were equalized to provide an accurate measure of the relative protein distribution. (B) Frozen and thawed mitochondria and submitochondrial particles (0.25 mg/mL) isolated from rat heart were incubated in 10 mM MOPS and 120 mM KCl (pH 7.4) for 5.0 min at 25 °C. Membrane fractions were then pelleted, and cytochrome *c* present in the supernatant (S) relative to total (T) (pellet + supernatant) was assessed by Western blot analysis using the anti-cytochrome *c* antibody.

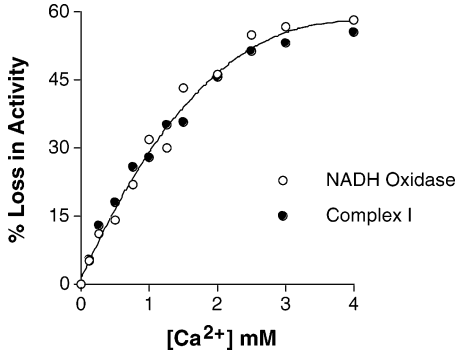

FIGURE 2: Concentration-dependent inhibition of complex I and NADH oxidase activities by Ca^{2+}. Submitochondrial particles (25 μg/mL) isolated from rat hearts were preincubated in 10 mM MOPS (pH 7.4) at 37 °C for 20 min with concentrations of Ca^{2+} indicated on the abscissa. This was followed by equilibration at 25 °C for 5.0 min. Complex I activity was monitored spectrophotometrically as the rate of NADH oxidation at 25 °C in 10 mM MOPS and 50 mM KCl (pH 7.4) upon addition of antimycin A (50 nM), ubiquinone-1 (100 μM), and finally NADH (100 μM) to initiate enzyme catalysis. NADH oxidase activity was measured in 10 mM MOPS and 50 mM KCl (pH 7.4) upon addition of 200 μM NADH in the absence of antimycin A and ubiquinone-1.

to $O_2^{\bullet-}$ production. MnSOD and the Krebs cycle enzyme α-ketoglutarate dehydrogenase (KGDH) were depleted in submitochondrial particles, indicating that $O_2^{\bullet-}$ scavenging and matrix enzymes had been effectively removed (Figure 1A). Treatment of frozen and thawed mitochondria with 120 mM KCl resulted in near-complete dissociation of cytochrome *c* from the inner mitochondrial membrane (Figure 1B). In contrast, treatment of submitochondrial particles with 120 mM KCl resulted in the minimal release of cytochrome *c* (Figure 1B), indicating that the submitochondrial particles were inside-out vesicles with cytochrome *c* trapped within and electron transport chain components accessible to electron donors NADH, succinate, and ubiquinol.

Effect of Ca^{2+} on Electron Transport Chain Complex Activities. The effects of Ca^{2+} on electron transport chain activities were assessed utilizing submitochondrial particles preincubated with varying concentrations of Ca^{2+} for 20 min at 37 °C. As shown in Figure 2, complex I activity was inhibited by Ca^{2+} in a concentration-dependent manner. Half-maximal inhibition was achieved at approximately 1.0 mM Ca^{2+} under the conditions of our experiments. The magnitude of complex I inhibition at various Ca^{2+} concentrations was not dependent on protein concentration over the range that was tested (5.0−100 μg of protein/mL). Incubation of submitochondrial particles for 20 min at 37 °C in the absence of Ca^{2+} did not induce appreciable alterations in the basal activity of complex I but resulted in a significant increase in the magnitude of complex I inhibition in the presence of Ca^{2+} (>3-fold). To investigate the specificity of Ca^{2+}-induced inhibition of complex I, the effects of Ca^{2+} on activities of other electron transport chain components were assessed. Ca^{2+} had little effect on complex II activity or on the combined activities of complexes III and IV (Table 1). Thus, Ca^{2+} acted specifically on complex I. The effects of complex I inhibition on overall electron transport chain activity and superoxide anion production were investigated.

Effect of Ca^{2+} on NADH and Succinate Oxidase Activities. The magnitude- and concentration-dependent inhibition of NADH oxidase by Ca^{2+} was similar to that observed for complex I (Figure 2). In contrast, Ca^{2+} exerted little appreciable effect on succinate-supported electron transport activity (complexes II−IV) (Table 1). Thus, Ca^{2+} acts specifically on complex I, reducing the overall rate of NADH-supported respiration or NADH oxidase activity. Treatment of submitochondrial particles with EGTA prior to enzyme analyses resulted in reactivation of complex I and NADH oxidase activity. Addition of EGTA after the initiation of enzyme activity failed to restore activity. In addition, inclusion of *N*-acetylcysteine or CuZnSOD in the presence or absence of EGTA did not prevent or restore enzyme activity (results not shown). Thus, unlike our previous findings for which frozen and thawed mitochondria were used (*47*), inhibition does not appear to be due to Ca^{2+}-induced $O_2^{\bullet-}$-dependent modification of complex I. Inhibition likely involves direct interaction of Ca^{2+} with complex I, an interaction that is stabilized upon enzymatic turnover.

Characterization of the Superoxide Anion Assay. We next sought to determine the effect of Ca^{2+}-induced complex I inhibition on the rate of $O_2^{\bullet-}$ production. Among numerous documented protocols and commercially available fluorescent probes (*20*), hydroethidine was used for quantitative assessment of the rates of NADH- and succinate-dependent $O_2^{\bullet-}$ production. This probe was chosen because (1) $O_2^{\bullet-}$-dependent formation of fluorescent 2-hydroxyethidium (*51*, *52*) is rapid, allowing assessment of the rate of $O_2^{\bullet-}$ production, (2) the fluorescence properties of 2-hydroxyethidium do not overlap with mitochondrial or substrate fluorescence, (3) exogenously added catalysts (e.g., horseradish peroxidase for the majority of H_2O_2 measurements) are not required, (4) the rate of $O_2^{\bullet-}$ production is dependent on the presence of substrate (Figure 3A) and exhibits a linear dependency on submitochondrial particle concentration (Figure 3B), (5) detection of $O_2^{\bullet-}$ production is blocked by CuZnSOD (Figure 3C,D), and (6) 2-hydroxyethidium fluorescence is insensitive to Ca^{2+} (Figure 3C) or electron transport chain inhibitors utilized in this study (not shown). Finally, on the basis of incubation conditions utilized in this study, subfractionation of hydroethidine and its fluorescent

Table 1: Effects of Ca^{2+} on Electron Transport Chain Activities[a]

	0 mM Ca^{2+}	2.0 mM Ca^{2+}
complex I (nmol of NADH min^{-1} mg^{-1})	402.2 ± 23.2[b]	213.4 ± 17.4[b]
complex II (nmol of ubiquinone-1 min^{-1} mg^{-1})	513.8 ± 17.2	464.3 ± 15.2
complexes III and IV (nmol of ubiquinol-1 min^{-1} mg^{-1})	742.8 ± 26.9	758.4 ± 26.6
NADH oxidase (nmol of NADH min^{-1} mg^{-1})	1219.8 ± 36.1[c]	676.7 ± 26.6[c]
succinate oxidase (nmol of O min^{-1} mg^{-1})	401.0 ± 88.5	378.7 ± 42.5

[a] Values represent the mean ± the standard deviation ($n = 5$). p values were determined from a paired t test. [b] $p < 10^{-6}$ between these two values. [c] $p < 10^{-8}$ between these two values.

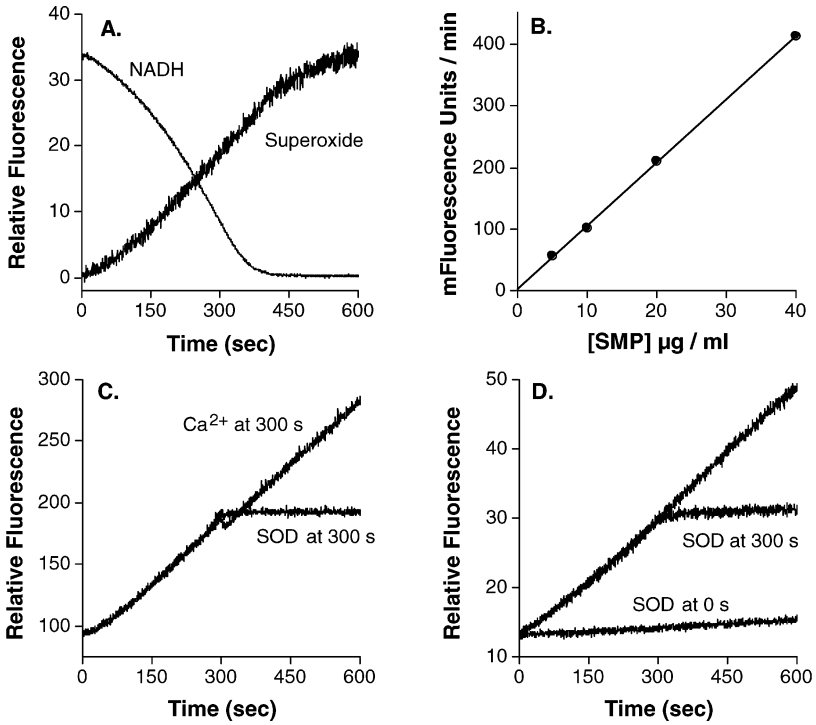

FIGURE 3: Characterization of superoxide anion measurements. (A) Submitochondrial particles (25 μg/mL) isolated from rat hearts were incubated in 10 mM MOPS and 50 mM KCl (pH 7.4) at 25 °C with 100 μM NADH. The rate of NADH consumption was measured fluorometrically (excitation and emission at 340 and 430 nm, respectively). The rate of NADH-dependent superoxide production was measured fluorometrically (excitation and emission at 480 and 567 nm, respectively) upon addition of 5.0 μM hydroethidine. (B) NADH-dependent superoxide production was assessed with submitochondrial particle protein concentrations given on the abscissa. (C) Xanthine oxidase (25 milliunits/mL) and xanthine (0.5 mM) were incubated in 10 mM MOPS and 50 mM KCl (pH 7.4) at 25 °C with 5.0 μM hydroethidine. The rate of superoxide production was measured fluorometrically (excitation and emission at 480 and 567 nm, respectively). At 300 s, Ca^{2+} (2.0 mM) or CuZnSOD (8.0 units/mL) was added as indicated. (D) Submitochondrial particles (25 μg/mL) isolated from rat hearts were incubated in 10 mM MOPS and 50 mM KCl (pH 7.4) at 25 °C with 100 μM NADH. The rate of NADH-dependent superoxide production was measured fluorometrically (excitation and emission at 480 and 567 nm, respectively) upon addition of 5.0 μM hydroethidine. At 0 or 300 s, CuZnSOD (8.0 units/mL) was added as indicated.

product (not shown), and the SOD-sensitive nature of $O_2^{\bullet-}$ detection (Figure 3C,D), $O_2^{\bullet-}$ released from the submitochondrial particles (matrix side in intact mitochondria) is exclusively detected.

Modulation of Superoxide Anion Production by Ca^{2+}. The effects of Ca^{2+}-mediated inhibition of complex I on NADH-supported $O_2^{\bullet-}$ production were evaluated. In contrast to the vast majority of electron transport chain inhibitors, Ca^{2+} reduced the basal rate of $O_2^{\bullet-}$ production (Figures 4 and 5A) by a magnitude consistent with the degree of complex I and NADH oxidase inhibition. To determine whether Ca^{2+} inhibits $O_2^{\bullet-}$ production promoted by downstream inhibition of electron transport, the complex III inhibitor antimycin A (prevents electron flow from cyt b_H to CoQ of the Q-cycle) was employed. As expected, near-complete inhibition of complex III with antimycin A stimulated production of $O_2^{\bullet-}$ via basal rates from 15.6 to 35.6 nmol of $O_2^{\bullet-}$ min^{-1} mg^{-1} (Table 2 and Figure 5B). Importantly, Ca^{2+} suppressed antimycin A-stimulated $O_2^{\bullet-}$ production by a magnitude consistent with the degree of complex I inhibition. Thus, the results indicate that Ca^{2+}-induced inhibition of complex I occurs upstream of sites of free radical production.

To further explore sites of free radical production and the effects of Ca^{2+}-induced complex I inhibition, additional electron transport chain inhibitors and substrates were employed. Myxothiazol, which inhibits the flow of electrons from $CoQH_2$ to the iron−sulfur protein within the Q-cycle of complex III (*53*−*56*), enhanced the rate of NADH-induced $O_2^{\bullet-}$ production, showing striking resemblance to the traces with antimycin A (Table 2 and Figures 5B,C). Moreover, Ca^{2+} suppressed myxothiazol-stimulated $O_2^{\bullet-}$ production (Table 2 and Figure 5C). Since myxothiazol is generally considered to inhibit before sites of free radical production within complex III (*53*−*58*), these findings indicate that the primary source of $O_2^{\bullet-}$ extruded from the submitochondrial particles is before complex III. In support of this conclusion,

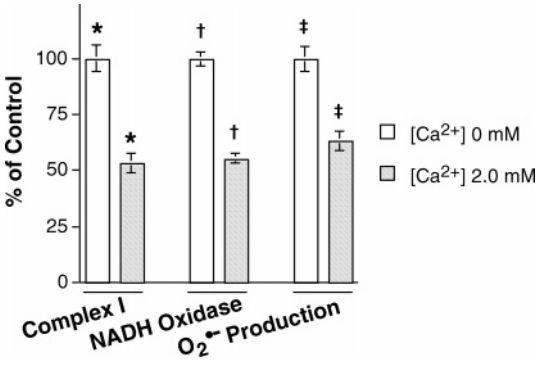

FIGURE 4: Effect of Ca^{2+} on complex I and NADH oxidase activities and the rate of superoxide anion production. Submitochondrial particles (25 μg/mL) isolated from rat hearts were preincubated in the absence and presence of 2.0 mM Ca^{2+} in 10 mM MOPS (pH 7.4) at 37 °C for 20 min. After a 5.0 min equilibration period at 25 °C, complex I activity and NADH oxidase activity were measured using 50 nM antimycin A, 100 μM ubiquinone-1, and 100 μM NADH or 200 μM NADH, respectively, in 10 mM MOPS and 50 mM KCl (pH 7.4). The rate of NADH-dependent superoxide production was measured fluorometrically (excitation and emission at 480 and 567 nm, respectively) upon addition of 5.0 μM hydroethidine and 500 μM NADH in 10 mM MOPS and 50 mM KCl (pH 7.4). Each data point represents the mean and standard deviation of five separate experiments [**(*)** $p < 10^{-6}$, **(†)** $p < 10^{-8}$, and **(‡)** $p < 10^{-5}$].

Ca^{2+} diminished the rate of basal and antimycin A- and myxothiazol-stimulated $O_2^{\bullet-}$ production when succinate was utilized as a respiratory substrate (Table 2). Because complex I is specifically inhibited by Ca^{2+} (Table 1), these results suggest that, with succinate, $O_2^{\bullet-}$ is produced by the flow of electrons from complex II into complex I, a process enhanced by inhibition of flow through complex III when antimycin A or myxothiazol is present.

DISCUSSION

This study employed submitochondrial particles in the evaluation of the effect of Ca^{2+} on electron transport chain activity and the rate of superoxide anion production. We provide evidence that complex I of the mitochondrial respiratory chain undergoes Ca^{2+}-induced inactivation. Inactivation was specific to complex I with no appreciable alterations in the activities of other electron transport chain complexes. The magnitude- and concentration-dependent nature of inactivation of complex I by Ca^{2+} was similar to that of the reduction in NADH-supported electron transport activity, indicating that complex I limits the rate of respiration. More importantly, the rate of $O_2^{\bullet-}$ production was suppressed by Ca^{2+}-induced inhibition of complex I. In addition, while inhibition of complex III stimulated $O_2^{\bullet-}$ production, Ca^{2+} reduced the relative rate of $O_2^{\bullet-}$ production, consistent with the magnitude of complex I inhibition. Ca^{2+} therefore inhibits electron transport upstream of the primary site(s) of free radical production. Inhibition of complex I by Ca^{2+} may represent a means for reducing the level of free radical generation and oxidative inactivation of matrix components.

Consistent with previous reports (*59*−*63*), evidence indicates that complex I is a primary source of $O_2^{\bullet-}$ released from submitochondrial particles or in the direction corresponding to the matrix side of the inner mitochondrial membrane. (1) Myxothiazol and antimycin A, which block electron transport before and after known sites of free radical generation within complex III (*53*−*58*), respectively, induce the same relative increase in the rate of $O_2^{\bullet-}$ production, and (2) succinate-induced $O_2^{\bullet-}$ production in the presence or absence of complex III inhibitors was suppressed by specific inhibition of complex I by Ca^{2+}, indicating that backflow into complex I was the primary source of $O_2^{\bullet-}$. While this is not universally observed (*64*), it has previously been shown that in uncoupled submitochondrial particles, succinate can support reverse electron flow to the N2 center of complex I (*65*). Rotenone, which inhibits complex I by interacting close to the ubiquinone binding site (*57*, *66*−*68*), did not reduce the rate of succinate-supported $O_2^{\bullet-}$ production (not shown). In contrast, rotenone abolished NADH-supported antimycin A- and myxothiazol-stimulated $O_2^{\bullet-}$ production (not shown). Our results suggest that, under the conditions of the experiments, $O_2^{\bullet-}$ is produced near the N2 center of complex I and may involve ubisemiquinone (*69*−*73*).

The ability of Ca^{2+} to interact with and inactivate complex I appears to require significant structural alterations to the protein. Structural rearrangements of complex I that provide access to Ca^{2+} likely underlie inactivation promoted by preincubation at 37 °C. This may involve the transition of complex I from the active to deactive state known to sensitize the enzyme to inhibition by divalent cations (*74*−*77*). Addition of EGTA prior to enzymatic turnover prevented complex I inhibition. In contrast, once enzymatic activity was initiated, EGTA failed to induce reactivation. These results suggest that enzyme-driven structural rearrangements prevent the subsequent release of Ca^{2+}. Because the degree of complex I inhibition depends on Ca^{2+} concentration but not on protein concentration, the initial binding affinity appears to be weak relative to expected physiological levels of Ca^{2+}. Previously, we demonstrated that in frozen and thawed mitochondria, complex I can undergo Ca^{2+}-mediated $O_2^{\bullet-}$-dependent inhibition due to oxidative modification of a protein-associated sulfhydryl(s) (*47*). In the study presented here, endogenously generated $O_2^{\bullet-}$ does not appear to be required for Ca^{2+}-mediated complex I inhibition. The physiological or pathophysiological significance of and relationship between these modes of Ca^{2+}-induced inactivation remain to be established. Nevertheless, the current mode of complex I inhibition requires Ca^{2+} concentrations 50-fold greater than those previously observed for Ca^{2+}-mediated $O_2^{\bullet-}$-induced inhibition (*47*). Given the importance of structural aspects to enzyme inactivation, this difference may represent environmental or architectural differences between submitochondrial particles and frozen and thawed mitochondria. In addition, components lost upon isolation of submitochondrial particles may be required for $O_2^{\bullet-}$-induced inhibition. Importantly, Ca^{2+}-induced $O_2^{\bullet-}$-independent inactivation may represent the initial component of $O_2^{\bullet-}$-dependent inhibition of complex I. Resolution of differences in Ca^{2+} concentrations required for each system may provide mechanistic insight into the overall process or indicate the existence of distinct modes of inhibition regulated by unique environmental alterations. Understanding conditions that govern the affinity of complex I for Ca^{2+} in its active and inhibited state, and, thus, the potential for reversible Ca^{2+}-mediated alterations in enzyme activity in response to

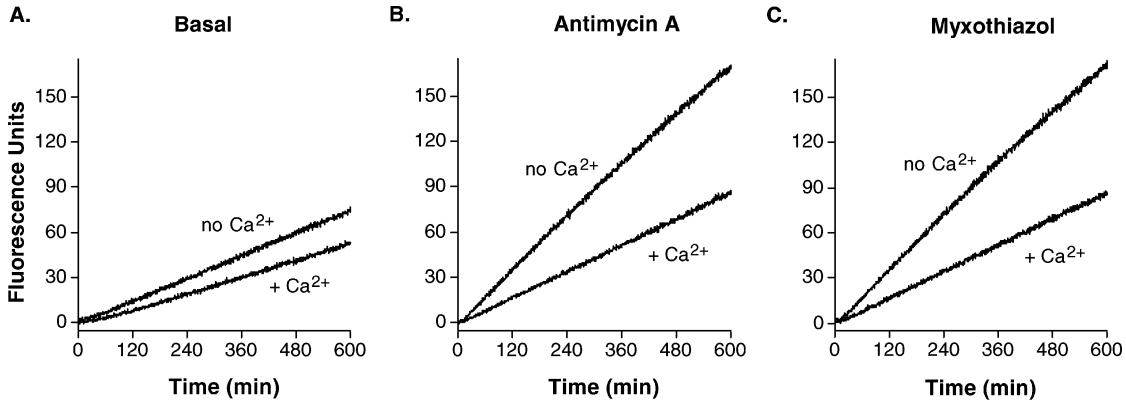

FIGURE 5: Effects of Ca^{2+} on the rates of antimycin A- and myxothiazol-stimulated superoxide anion production. Submitochondrial particles (25 μg/mL) isolated from rat hearts were preincubated in the absence and presence of 2.0 mM Ca^{2+} in 10 mM MOPS (pH 7.4) at 37 °C for 20 min. After a 5.0 min equilibration period at 25 °C, 5.0 μM hydroethidine and 500 μM NADH were added and fluorescence signals were recorded (excitation and emission at 480 and 567 nm, respectively) over time in 10 mM MOPS and 50 mM KCl (pH 7.4) in the absence and presence of (A) complex III inhibitors, (B) antimycin A, or (C) myxothiazol. CuZnSOD (8.0 units/mL) insensitive increases in fluorescence were subtracted from the traces that are presented.

Table 2: Effects of Ca^{2+} and Electron Transport Inhibitors on $O_2^{\bullet-}$ Production Rates[a]

	none	antimycin A	myxothiazol
NADH-supported			
0 mM Ca^{2+}	15.6 ± 0.4^b	35.6 ± 2.5^c	38.2 ± 1.4^d
2.0 mM Ca^{2+}	9.4 ± 0.3^b	17.0 ± 2.1^c	17.3 ± 0.1^d
succinate-supported			
0 mM Ca^{2+}	32.3 ± 1.0^e	42.1 ± 0.7^f	46.1 ± 0.1^g
2.0 mM Ca^{2+}	21.0 ± 0.4^e	20.0 ± 0.7^f	24.2 ± 0.1^g

[a] Values are expressed as nanomoles of $O_2^{\bullet-}$ per minute per milligram of protein and represent the mean $\pm$ the standard deviation ($n = 3$). p values were determined from a paired t test. [b] $p < 0.00005$ between these two values. [c] $p < 0.001$ between these two values. [d] $p < 0.002$ between these two values. [e] $p < 0.001$ between these two values. [f] $p < 10^{-5}$ between these two values. [g] $p < 10^{-6}$ between these two values.

(patho)physiological stimuli, is critical to defining the role of Ca^{2+} in the modulation of electron transport chain activity and $O_2^{\bullet-}$ generation.

There are a number of potential physiological and pathophysiological implications of Ca^{2+}-induced complex I inhibition. What emerges from this study is that the physiologically relevant divalent cation Ca^{2+}, known to increase in concentration during a number of degenerative processes associated with oxidative stress such as ischemia/reperfusion (*78*−*83*), can reduce the rate of $O_2^{\bullet-}$ production by the electron transport chain. This may represent an antioxidant response under conditions that promote free radical production by a compromised electron transport chain. Additionally, Ca^{2+} uptake by mitochondria is dependent on the proton gradient created across the inner mitochondrial membrane (*1*, *3*, *5*, *78*, *84*, *85*). Ca^{2+}-induced inhibition of complex I would be expected to diminish the proton gradient, thus limiting Ca^{2+} overload and subsequent large-scale disruption of mitochondrial structure and function. The significance of this study is the discovery that Ca^{2+} by itself can suppress $O_2^{\bullet-}$ production by specifically inhibiting complex I. The contribution of Ca^{2+}-induced inhibition of complex I to redox and Ca^{2+} homeostasis in response to physiological and pathophysiological stimuli as well as specific mechanistic aspects of this process remains to be fully delineated.

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