TOXICOLOGICAL SCIENCES **127(1)**, 110–119 (2012)
doi:10.1093/toxsci/kfs091
Advance Access publication February 17, 2012

Differential Inhibition of Electron Transport Chain Enzyme Complexes by Cadmium and Calcium in Isolated Rainbow Trout (*Oncorhynchus mykiss*) Hepatic Mitochondria

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Received December 20, 2011; accepted February 9, 2012

Impairment of the electron transport chain (ETC) is implicated in cadmium (Cd)- and calcium (Ca)-induced mitochondrial dysfunction. To localize the sites of the impairment, effects of 0–50μM Cd and Ca, singly and in combination, on complex I- to IV-driven respirations were investigated using isolated rainbow trout liver mitochondria. Mitochondrial Cd/Ca accumulation and respiration rates were measured following sequential inhibition and activation of complexes I, II, III, and IV. Mitochondrial adenosine triphosphate (ATP) synthesis was measured on exposure to (micromolar) 20 Cd and 50 Ca, singly and combined, whereas malondialdehyde (MDA) was measured on incubation with 0–1μM Cd and/or Ca. We show that mitochondrial accumulation of Cd and Ca and the states 3 and 4 rates of respiration depended on the active ETC complex. Although complex IV was highly recalcitrant to Cd and/or Ca, dose-dependent inhibitions of complex I-, II-, and III-driven state 3 respiration rates were observed with half maximal inhibitory concentrations (IC_{50}) of (micromolar) 12.4, 12, and 13.7 (Cd); 57.1, 46.1, and 26.2 (Ca); and 8.3, 13.5, and 5.1 (Cd + Ca), respectively. The lower IC_{50} values for complex I- and III-mediated respirations in the Cd + Ca treatment suggests that these complexes are the sites of cooperative actions of Cd and Ca. State 4 respiration rates were unaffected by Cd and/or Ca exposure but reduced mitochondrial coupling was apparent from the lower respiratory control and adenosine diphosphate/O ratios except in mitochondria oxidizing complex IV substrate. Additionally, there was reduced ATP synthesis in complex I substrates–energized mitochondria and increased MDA concentrations symptomatic of membrane lipid peroxidation.

Key Words: **cadmium; calcium; ETC complexes; ATP production; membrane peroxidation.**

Cadmium exposure in aquatic organisms has been shown to dysregulate cellular energy homeostasis (Adiele *et al*., 2011; Ivanina *et al*., 2008; Sokolova *et al*., 2005). Because most of the cellular energy requirement is produced by the mitochondria (Nelson and Cox, 2009), impairment of these organelles is fundamental for aberrant energy balance. A prerequisite for mitochondrial impairment by Cd is that the cation must bind to or transverse the selectively permeable inner mitochondrial membrane (IMM) to harm structures involved in oxidative phosphorylation (OXPHOS). In OXPHOS, transfer of electrons in the electron transport chain (ETC) is coupled to transport of protons from the matrix into the intermembrane space creating an electrochemical gradient across the IMM. The energy generated by this gradient is subsequently used by the terminal ETC enzyme complex (F_1F_0-ATPase) to synthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate. Impaired OXPHOS results in energy deficits to support vital physiological processes such as the synthesis and maintenance of essential cellular macromolecules (Buttgereit and Brand, 1995). Although previous studies in isolated mammalian (Belyaeva and Korotkov, 2003; Dorta *et al*., 2003; Reynafarje and Ferreira, 2008) and fish (Adiele *et al*., 2012) mitochondria have shown that Cd impairs the ETC, the specific components of the chain affected have not been unambiguously identified. Moreover, the potential that individual ETC enzyme complexes are unequally sensitive to Cd is yet to be explored in fish mitochondria given the demonstration that the sensitivity of ETC complexes in eastern oysters (*Crassostrea virginica*) differs from that of rats (Ivanina *et al*., 2008).

Unlike Cd, Ca is an essential element participating in multiple signaling cascades that maintain intracellular homeostasis (Brooke *et al*., 2004; McCormack and Denton, 1990). Fluctuations in intracellular Ca concentrations stimulate mitochondria either to sequester or release Ca (Gunter *et al*., 1994). The physiological effects of Ca in the mitochondria include stimulation of the substrate oxidation and phosphorylation subsystems by activating matrix dehydrogenases (McCormack and Denton, 1993), F_1F_0-ATPase synthase (Das and Harris, 1990), and adenine nucleotide translocase (Mildaziene *et al*., 1995). Additionally, uptake of Ca by the mitochondria contributes to the maintenance of cellular Ca homeostasis (Gunter *et al*., 1994). Regardless, when Ca is present at elevated concentrations, it induces toxicity through actions at multiple

cellular locations. At the mitochondrial level, high Ca stimulates degradative enzymes including calpain proteases and phospholipases exacerbating its direct deleterious effects on mitochondrial bioenergetics (Nakahara *et al*., 1992; Wingrave *et al*., 2003). Further, elevated cytosolic Ca concentration promotes mitochondrial permeability transition and swelling (Adiele *et al*., 2012; Gunter *et al*., 1994) with dissipation of membrane potential (Brooke *et al*., 2004) and free radical synthesis (Starkov *et al*., 2004). Because of similar physicochemical characteristics, Ca homeostasis is dysregulated by Cd to enhance deleterious effects of Cd (Gunter *et al*., 1994). Thus, it is of interest to investigate the interactive effects of Cd and Ca on mitochondrial function.

An estimated 1–2% of the O_2 consumed by the mitochondria is converted to reactive oxygen species (ROS) (Turrens, 2003; Wang *et al*., 2004). Specifically, mitochondrial redox carriers inadvertently release lone electrons that partially reduce O_2 with formation of superoxide anion radical ($O_2^{\bullet -}$) (Turrens, 2003). The $O_2^{\bullet -}$ then spontaneously or enzymatically dismutates into hydrogen peroxide (H_2O_2) and O_2, with the H_2O_2 being reduced to H_2O. Alternatively, the $O_2^{\bullet -}$ attacks mitochondrial proteins bearing iron-sulfur (Fe-S) centers releasing Fe^{2+} that participate in Fenton reactions with conversion of H_2O_2 into hydroxyl ions and hydroxyl radicals (Raha and Robinson, 2000). When in excess the ROS oxidize proteins, DNA and membrane lipids (Kohen and Nyska, 2002). Although previous studies suggest that complexes I and III are the primary sites of ROS production in the mitochondria (Li *et al*., 2003; Turrens, 2003), there have been no studies in fish to assess whether the ETC complexes associated with ROS production are impacted by Cd and/or Ca to induce oxidative damage.

The aim of the present study was to localize the sites of deleterious actions of Cd and Ca in rainbow trout liver mitochondria (RTLM) ETC. We predicted that sequential inhibition of the ETC enzyme complexes coupled with the use of substrates that deliver electrons to different points of the ETC would reveal not only the most sensitive region in the chain but also the sites of cooperative actions of Cd and Ca previously demonstrated (Adiele *et al*., 2010). Additionally, we hypothesized that Cd- and/or Ca-induced impairment of the ETC would impair ATP synthesis and promote generation of ROS leading to mitochondrial membrane lipid peroxidation with elevated concentrations of a biomarker of oxidative stress, malondialdehyde (MDA).

MATERIALS AND METHODS

Fish. Rainbow trout (*Oncorhynchus mykiss*) purchased from Ocean Trout Farm Inc., Brookvale, Canada, were held in a 250-l tank with flow-through aerated well water at the Atlantic Veterinary College Aquatic Animal Facility. Major ion concentrations in milligram per liter were: Na 47, Ca 59, Mg 28, K 2.3, Cl 137, SO_4 17, hardness (as $CaCO_3$) 260, and total alkalinity (as $CaCO_3$) 145. The water temperature range was 10–11°C, and pH was 7.5. Fish were fed *ad libitum* on alternate days with 3 mm^3 trout chow pellets (Corey Feed Mills, Fredericton, Canada) containing, as specified by the manufacturer: crude protein 50.0% (minimum), crude fat 20.0% (minimum), crude fiber 1.4% (maximum), calcium 1.7% (17 mg/g actual), phosphorus 1.0% (actual), sodium 0.6% (actual), vitamin A 2500 IU/kg (minimum), vitamin D_3 2400 IU/kg (minimum), and vitamin E 200 IU/kg (minimum). Cadmium background concentrations measured in feed and water were (0.78 μg/g) and below our limit of detection (0.03 μg/l), respectively.

Mitochondria isolation. Mitochondria were isolated from the liver according to Adiele *et al*. (2010). Rainbow trout (mean fish weight = 202 ± 32 g) were sacrificed by a blow to the head. Livers were immediately dissected out, rinsed with ice-cold mitochondria isolation buffer (MIB: 250mM sucrose, 10mM Tris-HCl, 10mM KH_2PO_4, 0.5mM EGTA, 1 mg/ml bovine serum a*lbumin (*BSA) [fatty acid free], 2 μg/ml aprotinin, pH 7.3), and blotted dry. Each liver was weighed, sliced into small pieces, and homogenized by five turns of a loosely fitting Teflon pestle in three volumes of ice-cold MIB using 10-ml Potter-Elvehjem homogenizer (Cole Parmer, Anjou, Canada) on ice. The crude liver homogenates were transferred to 1.5 ml tubes and centrifuged at 800 × g, 4°C for 15 min. The supernatants were transferred to fresh centrifuge tubes for further centrifugation at 13,000 × g, 4°C for 10 min to get mitochondrial pellets. Each pellet was subsequently washed twice by resuspending in ice-cold MIB with centrifugation at 11,000 × g, 4°C for 10 min. The final mitochondrial pellets were weighed and resuspended in 3 volumes of EGTA-free ice-cold-mitochondrial respiration buffer (MRB: 10mM Tris-HCl, 25mM KH_2PO_4, 100mM KCl, 1 mg/ml BSA [fatty acid free], 2 μg/ml aprotinin, pH 7.3) and used for the respiratory experiments within 4 h after isolation while being kept on ice. In our earlier study, mitochondrial functional attributes remained stable within this time interval (Adiele *et al*., 2010). Effects of Cd and Ca, singly and in combination, in the presence or absence of the inhibitors of the ETC complexes and complex-specific substrates were studied using mitochondrial suspensions from five different fish ($n = 5$). To reduce chelation of cations that would reduce their bioavailability for uptake and binding to sensitive mitochondrial sites, EGTA was omitted in the MRB. BSA also binds Cd but was retained in the buffer to maintain mitochondrial integrity (DelRaso *et al*., 2003; Kurochkin *et al*., 2011).

Mitochondrial respiration. Complex I–IV substrate–energized mitochondrial respiration rates (oxygen consumption; nmol O_2/mg protein/min) were measured with Clark-type oxygen electrodes (Qubit Systems, Kingston, Canada) housed in 1.5-ml cuvettes. The cuvettes were preloaded with 1.45 ml of MRB under continuous stirring. The temperature was maintained at 15°C using a circulating cooling water-bath (Haake, Karlsruhe, Germany). The protein concentrations of mitochondrial samples were initially measured spectrophotometrically (Spectramax Plus 384; Molecular Device, Sunnyvale, CA) by the Bradford (1976) method. Thereafter, 100 μl of mitochondrial suspensions containing 1.7–1.9 mg protein were introduced into the oxidation cuvettes followed by ETC inhibitors (0.5μM rotenone [complex I], 25μM malonate [complex II], or 20nM antimycin A [complex III]) and substrates (5mM malate-glutamate [complex I], 5mM succinate [complex II], 3μM reduced decylubiquinone [decylubiquinol, reduced with sodium borohydride ($NaBH_4$)] [complex III], or 5mM ascorbate + 200μM TMPD [complex IV]) using Hamilton syringes fitted with 6.35 cm needles. Single optimal concentrations of the inhibitors and substrates that did not impair mitochondrial respiration were determined by initial preliminary dose-response studies. For all the complexes, addition of 375 nmol of ADP invoked the highest rate of mitochondrial oxygen consumption (ADP-stimulated/state 3 respiration), which eventually transitioned to state 4 respiration upon depletion of the ADP (ADP-limited respiration rate). The effects of Cd and Ca, singly and in combination, on ETC enzyme complexes I- to IV-driven respirations were investigated using four nominal concentrations (5, 10, 20, and 50μM) of Cd (as $Cd[NO_3]_2 \bullet 4H_2O$, Sigma-Aldrich, Oakville, Canada) and Ca (as [$CaCl_2 \bullet 2H_2O$, Sigma-Aldrich]). The actual concentrations of Cd measured by graphite furnace atomic absorption spectrometry (GFAAS, AAnalyst 800, Perkin Elmer, Foster City, CA) were in micromolar 4, 11, 18, and 48, respectively, whereas the actual Ca concentrations measured by flame atomic absorption spectrometry (AAS) were (micromolar): 5, 11, 22, and 51, respectively. For the combined treatment,

respective equimolar concentrations of Cd and Ca were added simultaneously to actively phosphorylating mitochondria in complete assay mixtures containing ADP, inhibitors, and substrates of ETC complexes. States 3 and 4 rates of respiration were recorded with LabPro data acquisition software (Qubit Systems) and normalized to mitochondrial protein. The respiratory control ratio (RCR) was calculated according to Estabrook (1967), whereas the ADP/O ratio (phosphorylation efficiency) was calculated as the amount of ADP added divided by the total amount of oxygen consumed as atoms (Chance and Williams, 1955). Prior to all measurements of respiration, the electrodes were calibrated at 0 mg O_2/l (0% O_2) and air saturated Milli-Q (Millipore, Bedford, MA) water at ambient atmospheric pressure (734–759 mmHg) measured by a traceable digital barometer (Fisher Scientific, Nepean, Canada).

Cadmium and Ca analysis. Measurement of mitochondrial Cd and Ca accumulation was done by AAS. Upon completion of the respiration measurements, mitochondrial suspensions were centrifuged at 11,000 × g, 4°C for 5 min and the resulting pellets were washed by resuspending in 20 volumes of MRB and centrifuging at 11,000 × g, 4°C for 5 min to remove nonspecifically bound Cd and Ca ions. The pellets were then lyophilized, weighed, and digested with 200 µl of 30% H_2O_2 and 70% HNO_3 (trace metal grade, Fisher Scientific) in a 1:15 ratio for 48 h at room temperature. For Ca, lanthanum chloride was added to the mitochondrial digest, and the concentrations were measured using flame AAS and expressed as mmol Ca/g mitochondrial dry weight (mdw). A certified reference material SLRS-4 containing 6.2 ± 0.2 mg/l Ca (National Research Council of Canada, Ottawa, ON) was analyzed together with Ca samples. For Cd, appropriate dilutions of the digests were done with Milli-Q water, and Cd concentrations were measured by graphite furnace AAS and expressed as mmol Cd/g mdw. All Cd concentration measurements were done in the presence of a matrix modifier ($NH_4H_2PO_4$ and $Mg(NO_3)_2$). Standard reference materials (TMDA-54.4) containing 158 ± 2 µg/l Cd (National Water Research Institute, Burlington, Canada), Tort-2 containing 26.7 ± 0.6 µg/g Cd (National Research Council of Canada), and blanks were analyzed along with the samples. The blanks were below the Cd limit of detection (0.03 µg/l) and the recovery rates for SLRS-4, TMDA-54.4, and Tort-2 were (means ± SD, n = 5–8) 92 ± 10, 107 ± 4, and 96 ± 5%, respectively.

Mitochondrial ATP production. Mitochondrial rates of ATP production were measured by firefly luciferase assay according to the manufacturer's instructions using ATP bioluminescence assay kit (Sigma-Aldrich). Effects of nominal (actual) concentrations (micromolar) of 20 (18) Cd and 50 (51) Ca, singly and in combination, were measured using malate-glutamate energized mitochondrial (1.9–2.4 mg protein) suspension in 1.5 ml of MRB containing 375 nmol ADP. Proteins were precipitated by addition of 75 µl of 70% perchloric acid to 750 µl of the mitochondrial suspension. The supernatant was recovered by centrifugation at 11,000 × g, 4°C for 5 min and used for measurement of ATP concentration. This involved addition of 100 µl of ATP assay mix to the protein-free samples and ATP standards in 96-well plates, followed by monitoring of the luminescence signal (BioTek Instruments, Winooski, VT). The luminescence signal was then converted to ATP concentration using the generated standard curve and expressed as µmol ATP/mg protein/min.

MDA measurement. Mitochondrial membrane peroxidation was quantified by measuring MDA production using an MDA assay kit (Northwest Life Science, Vancouver, WA) according to manufacturer's instructions. Briefly, mitochondrial suspensions (0.7–0.8 mg protein) were incubated with 0–1µM of Cd and Ca, singly and in combination, on ice for 1 h. The suspensions were then sonicated (60 Sonic Dismembrator, Fisher Scientific) for 30 s and centrifuged at 10,000 × g, 4°C for 2 min. The resulting supernatants were transferred into fresh 1.5-ml centrifuge tubes and stored at −80°C until used. For the assay, 5 µl butylated hydroxytoluene in ethanol, 125 µl of 1M phosphoric acid, and 125 µl thiobarbituric acid reconstituted with 10.5 ml Milli-Q water were added to 125 µl of the supernatants and MDA standards. These were then vortexed for 10 s and incubated at 60°C for 1 h in a water-bath (ISOTEMP 205; Fisher Scientific, Dubuque, Iowa). Subsequently, the samples and standards were allowed to cool down at room temperature after which butanol (300 µl) was added and the mixture vortexed for 5 min. Samples were then centrifuged at 10,000 × g at room temperature for 3 min to separate the butanol fraction (upper pink layer). Subsequently, 1 N NaOH (250 µl) was added to 250 µl of the butanol fraction taken in a fresh tube and vortexed for 5 s to get the NaOH fraction (lower layer). Finally, 200 µl of the NaOH fraction was transferred to another fresh tube, and 50 µl of 3.7 N phosphoric acid was immediately added. After vortexing for 10 s, 200 µl of the reaction mixture was transferred to 96-well plate for spectrophotometry (Spectramax Plus 384; Molecular Device) to obtain spectra scan (400–700 nm). The concentration of MDA was then determined by derivative spectroscopy data analysis.

Statistical analysis. Data were checked for normality of distributions (Chi-Square test) and homogeneity of variances (Levene's test) and were found to be normally distributed and of homogeneous variances. The half maximal inhibitory concentrations (IC_{50}) for Cd, Ca, and Cd + Ca on state 3 respiration rate were calculated by four parameter logistic nonlinear regression analysis (SigmaPlot 10.0, Systat Software Inc., IL). The cation accumulation, mitochondrial respiration, IC_{50}, and MDA concentration data were submitted to two-way ANOVA (Statistica StatSoft Inc., Tulsa, OK), with treatment groups (Cd, Ca, and Cd + Ca exposures), exposure concentrations, and/or ETC complexes as independent variables. The ATP data were submitted to one-way ANOVA with cation exposure as the independent variable. *Post hoc* pair-wise comparisons were done using Tukey's honest significant difference test. All the data are reported as means ± SEM with a level of significance of $p < 0.05$.

RESULTS

Mitochondrial Cd and Ca Accumulation

During exposure to Cd alone, mitochondrial Cd accumulation was dose dependent ($F_{4,80} = 275.35$, $p < 0.0001$) and varied significantly ($F_{3,80} = 125.98$, $p < 0.0001$) with the activated ETC enzyme complex (Fig. 1a). Similarly, in the combined exposure, Cd accumulation increased dose dependently ($F_{4,80} = 99.80$, $p < 0.0001$) according to the active ETC complex ($F_{3,80} = 10.32$, $p < 0.0001$) (Fig. 1b). For Ca, significant accumulation occurred in mitochondria exposed to Ca alone ($F_{4,80} = 27.26$, $p < 0.0001$) (Fig. 2a) and in combination with Cd ($F_{4,80} = 22.72$, $p < 0.0001$) (Fig. 2b). This accumulation was not influenced by the active ETC complex in both the Ca ($F_{3,80} = 1.14$, $p = 0.34$) alone (Fig. 2a) or the combined ($F_{3,80} = 0.95$, $p = 0.42$) (Fig. 2b) exposures.

Mitochondrial Respiration

State 3 mitochondrial respiration varied according to the active ETC complex ($F_{3,80} = 31.51$, $p < 0.0001$) and was inhibited dose dependently by Cd exposure ($F_{4,80} = 74.05$, $p < 0.0001$). Maximal inhibitions were 65, 95, and 65% for respiration driven by the complex I, II, and III substrates used, respectively. The calculated Cd IC_{50} values for state 3 respiration rates were 12.4, 12, and 13.7µM for complexes I, II, and III, respectively (Table 1). Interestingly, complex IV-mediated state 3 rate of respiration was not affected by Cd exposure (Fig. 3a). Similarly on Ca alone exposure, state 3 respiration rates varied according to the activated ETC complex ($F_{3,80} = 9.60$, $p < 0.0001$) with a highly significant inhibitory effect of Ca ($F_{4,80} = 31.09$, $p < 0.0001$). There were 55, 50,

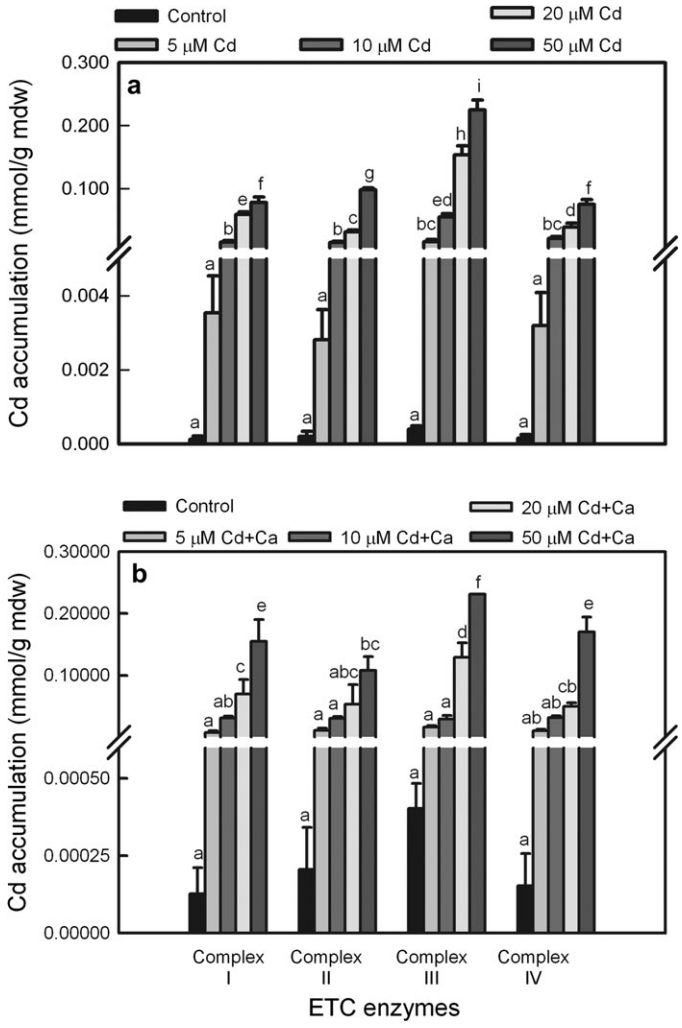

FIG. 1. Cadmium accumulation in isolated RTLM during respiration mediated by complexes I–IV of the ETC. The mitochondria were exposed to 0 (control), 5, 10, 20, and 50μM Cd singly (a) and in combination (b) with equimolar Ca concentrations. Data are means ± SEM ($n = 5$). Bars with dissimilar letters are significantly different from one another (ANOVA, $p < 0.05$).

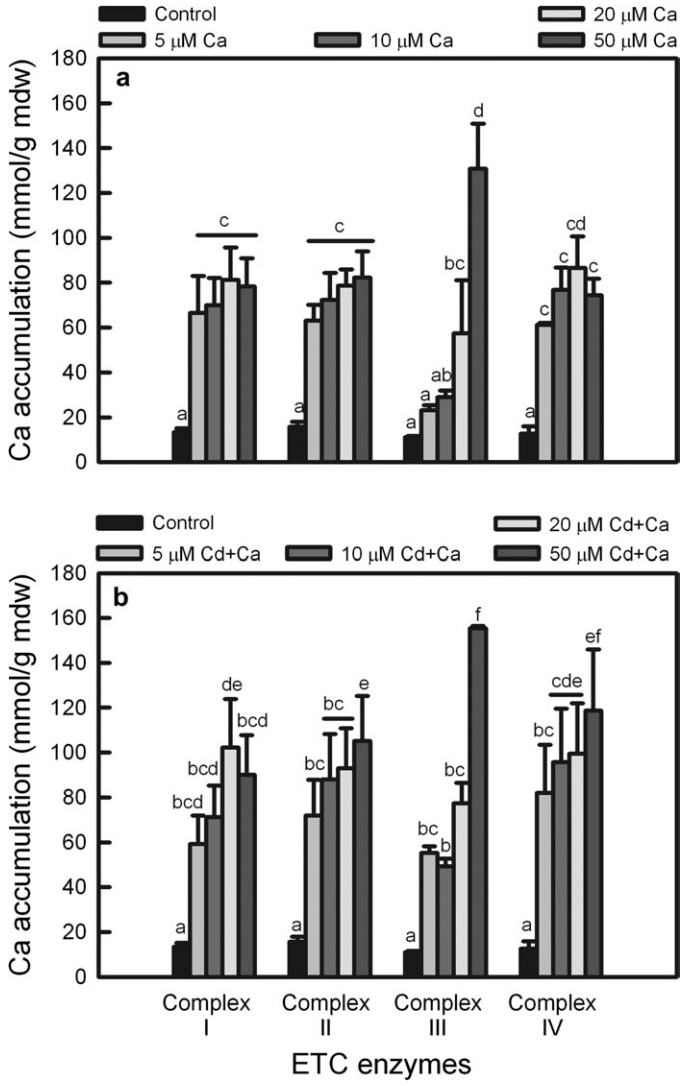

FIG. 2. Calcium accumulation in isolated RTLM during respiration mediated by complexes I–IV of the ETC. The mitochondria were exposed to 0 (control), 5, 10, 20, and 50μM Ca singly (a) and in combination (b) with equimolar Cd concentrations. Data are means ± SEM ($n = 5$). Bars with dissimilar letters are significantly different from one another (ANOVA, $p < 0.05$).

and 75% reductions in complex 1-, II-, and III-driven state 3 respiration rates representing IC_{50} values of 57.08, 46.09, and 26.19μM, respectively (Table 1). As with Cd, Ca alone did not have any effect on complex IV-driven state 3 respiration rate (Fig. 3b). Lastly, during exposure to Cd + Ca, state 3 respiration rates varied according to the active complex ($F_{3,80} = 58.70$, $p < 0.0001$) and were significantly impaired ($F_{4,80} = 44.57$, $p < 0.0001$) by the two cations combined. Maximal inhibitions were 80, 90, and 80% for respirations driven by the complex I, II, and III substrates used, respectively, corresponding to IC_{50} values of 8.3, 12.7, and 5.12μM, respectively (Table 1). Complex IV-driven respiration remained resistant to inhibitory effects of combined Cd and Ca exposure (Fig. 3c). Generally, state 3 respiration rates driven by complex I, II, and III all were negatively related to Cd and Ca accumulation in the respective single and combined cation exposures (Supplementary figs. S1a–d, S2a–d, and S3a–d).

State 4 respiration rates varied with the activated complex ($F_{2,60} = 34.80$, < 0.0001) but were not affected by Cd exposure ($F_{4,60} = 0.15$, $p = 0.96$). Complex IV-driven state 4 respiration was not estimated because of depletion of O_2 in the respiratory medium. Similarly, while state IV respiration rates varied significantly with the active complex ($F_{3,80} = 46.07$, $p < 0.0001$) during Ca exposure, in itself Ca had no effect ($F_{4,80} = 0.33$, $p = 0.86$). The rate of complex IV-driven state 4 respiration was higher than the respective rates mediated by complexes I–III (Table 2). Lastly, during exposure to Cd + Ca, complex I- to III-mediated state 4 respiration rates varied with the activated complex ($F_{2,60} = 17.99$, $p < 0.0001$) but the two

TABLE 1
Half Maximal Inhibitory Concentrations (IC_{50}) for Complex-Specific State 3 Respiration Rates

	Half maximum inhibitory concentrations (IC_{50}; μM) 15°C			
Exposure groups	Complex I	Complex II	Complex III	Complex IV
Cd	12.39 ± 1.12***	12.04 ± 0.18***	13.69 ± 2.09***	NE
Ca	57.08 ± 2.94******	46.09 ± 2.60*****	26.19 ± 2.95****	NE
Cd + Ca	8.30 ± 1.69**	13.50 ± 1.66***	5.14 ± 0.95*	NE

Notes. Isolated rainbow trout liver mitochondria were energized by ETC complex-specific substrates and exposed to 0–50μM Cd and Ca, singly and in combination, at 15°C. Values with different numbers of asterisks (*) are statistically different from one another (ANOVA, $p < 0.05$). Data are means ± SEM ($n = 5$). NE represents No Effect on ETC complex-mediated respiration.

cations combined had no effect on these rates ($F_{4,60} = 0.31$, $p = 0.87$). Complex IV-driven state 4 respiration was not calculated because it was not discernible due to O_2 depletion.

When mitochondria were exposed to Cd, the RCR varied with respect to activated ETC complex ($F_{2,60} = 5.33$, $p = 0.007$) and were significantly ($F_{4,60} = 11.35$, $p < 0.0001$) reduced (Table 3). Maximal reductions were 30, 15, and 40% for complex I-, II-, and III-driven respiration, respectively. Complex IV-derived RCR was not calculated due to inability to estimate state 4 rate of respiration. In the Ca exposure group, the RCR varied according to the active ETC complex ($F_{3,80} = 55.00$, $p < 0.0001$). Overall, Ca reduced the RCR ($F_{4,80} = 11.67$, $p < 0.0001$) with the highest effect (60% reduction) being observed for complex III-mediated respiration. The RCR also was significantly reduced on exposure to Cd + Ca ($F_{4,60} = 11.32$, $p < 0.0001$), but active complex had no effect ($F_{3,60} = 2.06$, $p = 0.14$). Overall, complex I-, II-, and III-derived RCRs were reduced by 40, 45, and 50%, respectively. The RCR for complex IV was not estimated due to inability to deduce the corresponding state 4 rate of respiration.

The phosphorylation efficiency (ADP/O ratio) (Table 4) varied according to the active ETC complex ($F_{2,60} = 15.23$, $p < 0.0001$) and was reduced significantly by Cd ($F_{4,60} = 4.75$, $p = 0.002$). The ADP/O ratio for oxidation of complex IV substrates was not estimated because the corresponding O_2 consumption was not estimated. In the Ca alone exposure, the ADP/O ratio varied with the active ETC complex ($F_{3,80} = 49.59$, $p < 0.0001$) with the cation having no effect ($F_{4,80} = 0.79$, $p = 0.54$). In contrast, the ADP/O ratios obtained following combined exposure to Cd and Ca did not vary with the active ETC complex ($F_{4,60} = 2.32$, $p = 0.107$) but were significantly reduced by the Cd + Ca treatment ($F_{4,60} = 3.17$, $p = 0.02$). Complex II ADP/O ratio was the most severely impacted and exhibited 50% reduction. The ADP/O ratio for oxidation of complex IV-driven respiration was not estimated due to inability to measure the corresponding state 4 rate of O_2 consumption.

ATP Production Rate and MDA Levels

The rate of ATP synthesis on exposure of RTLM to Cd, Ca, and Cd + Ca was significantly inhibited (treatment effect: $F_{3,16} = 54.30$, $p < 0.0001$) by 75, 60, and 85%, respectively (Fig. 4). For MDA, there was a significant dose effect ($F_{4,60} = 5.78$, $p = 0.0005$) on exposure to Cd, Ca, and Cd + Ca (Fig. 5), with the MDA concentrations increasing to the same level (3× the control values) in all of the treatment groups ($F_{2,60} = 0.14$, $p = 0.87$).

DISCUSSION

The present study demonstrates that Cd accumulation in isolated RTLM depends on the active ETC enzyme complex with the highest and lowest accumulation occurring during respiration fueled by complex III and I/IV substrates, respectively. Moreover, the substrate that generated the highest respiration rate resulted in higher Cd accumulation, reiterating the theme that the state of energization influences mitochondrial Cd permeation and accumulation (Adiele *et al*., 2010; Belyaeva *et al*., 2002). For Ca, accumulation was highest during complex III-mediated respiration, whereas mitochondria oxidizing complex I, II, and IV substrates accumulated lower and comparable amounts of Ca. Consistent with our previous findings (Adiele *et al*., 2010, 2012), Cd/Ca accumulation was enhanced in the combined exposure contrasting the antagonism that occurs at epithelia of external surfaces such as the gill (Niyogi and Wood, 2004). Here, we show that the enhancement of accumulation depends on the active ETC complex, being highest during complex II- and III-mediated respirations. Although the basis for the disparate Cd-Ca interactions observed in the mitochondria and the gill epithelium is not known, it may be related to membrane structure differences e.g., the cholesterol/phospholipid ratio which determines membrane protein integration and function (Bastiaanse *et al*., 1997). Notably, the IMM lipid composition with high levels of cardiolipin and virtually no cholesterol together with special transporters may result in unique interactions with toxicants.

Cadmium toxicity may result from its deleterious effects on components of mitochondrial subsystems (Brand, 1998; Ivanina *et al*., 2008; Kurochkin *et al*., 2011). Thiol ligands, nitrogen-containing functional groups, Fe-S clusters of the ETC complexes, and binding sites for essential divalent metals

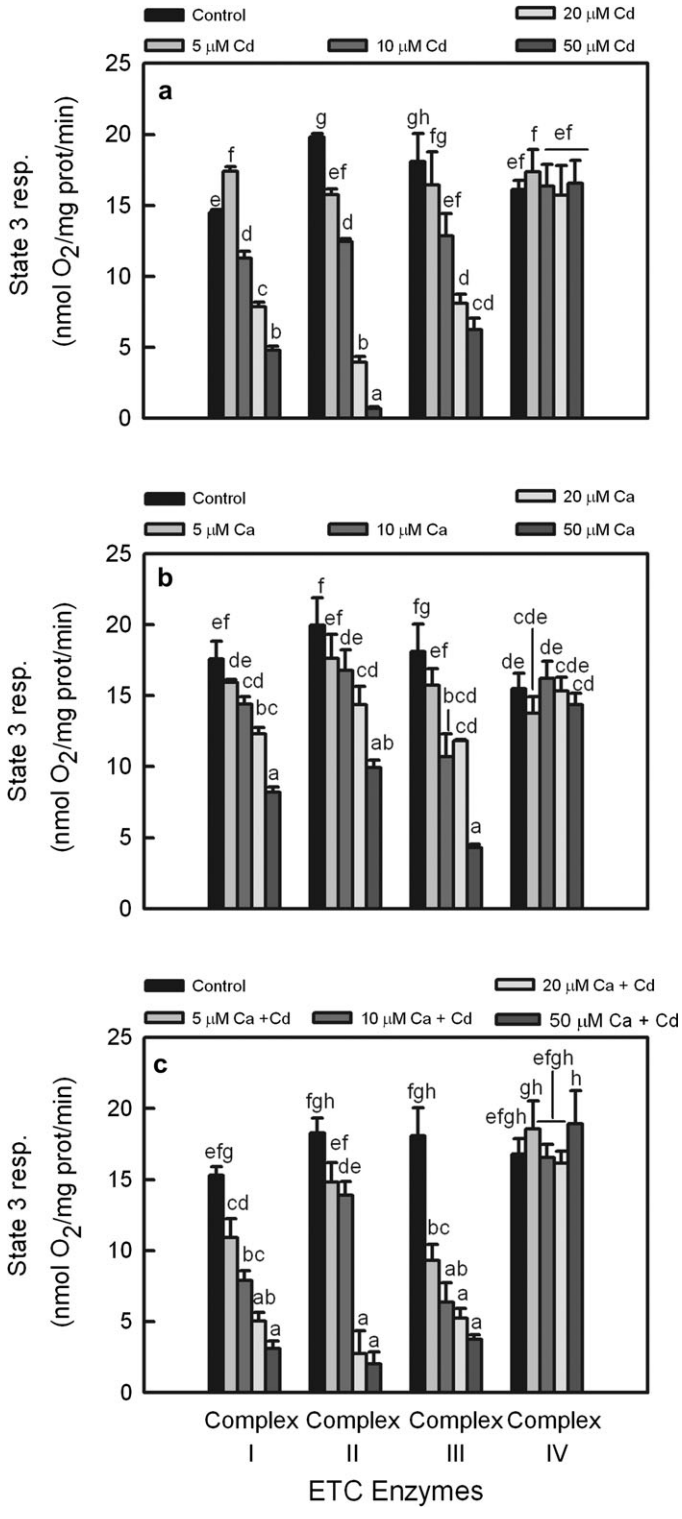

FIG. 3. State 3 respiration rates in isolated RTLM fueled by substrates for complexes I-IV of the ETC. The mitochondria were exposed to 0 (control), 5, 10, 20, and 50μM Cd (a), Ca (b) and respective equimolar concentrations of Cd + Ca (c). Data are means ± SEM ($n = 5$). Bars with dissimilar letters are significantly different from one another (ANOVA, $p < 0.05$).

in biomolecules constitute potential Cd targets sites (Dorta *et al*., 2003; Kurochkin *et al*., 2011). The outcome of the interactions of Cd with these target sites include inhibition of electron transfer through the ETC with dissipation of the proton-motive force necessary for OXPHOS (Kurochkin *et al*., 2011; Wang *et al*., 2004). Our study shows that Cd inhibits respiration driven by complexes I, II, and III concentration dependently, whereas complex IV-driven respiration is highly recalcitrant. Although the causes of the insensitivity of complex IV to Cd were not investigated, we speculate that structural differences among the complexes could have contributed. Specifically, the three complexes (I, II, and III) that are sensitive to Cd contain Fe-S clusters, whereas the nonresponsive complex IV does not.

The inhibitory potencies of Cd and Ca on the ETC enzyme complexes have rarely been studied in fish. While Garceau *et al*. (2010) showed that Cd at lower concentrations than those used in the present study enhanced complex IV (cytochrome c oxidase) activity in goldfish (*Carassius auratus*) liver mitochondria, to the best of our knowledge, the dose-response of Cd on mitochondrial respiration and the interactive effects with Ca have not been investigated in fish. In the present study, RTLM complexes I, II, and III exhibited comparable sensitivity to Cd based on inhibition of state 3 respiration rates (Table 1) while the order of sensitivity to Ca was complex III > complex II > complex I. In contrast, Sokolova (2004) measured a state 3 Cd IC_{50} of 80μM in eastern oyster hepatopancreatic mitochondria respiring on succinate at 15°C, a value approximately 7× higher than that measured in the present study for succinate-supported RTLM at the same temperature. Additionally in eastern oysters, the IC_{50} values for ETC enzyme complexes isolated from the hepatopancreas were (micromolar) 500 for complex II and > 1000 for complexes I, III, and IV, with complex III being the least sensitive (Ivanina *et al*., 2008). In rats the order of sensitivity of ETC enzyme complexes to Cd was shown to be complex III > complex II > complex I > complex IV (Wang *et al*., 2004). The variable ETC enzyme sensitivity to Cd is perhaps a reflection of species-specific differences in enzyme properties and/or structure.

Under experimental conditions using ETC complex-specific inhibitors and substrates, the components of the chain distal to the point of inhibition or electron entry (depending on substrate) are activated and participate in electron transport. Thus in mitochondria supported by glutamate-malate (complex I substrates), the entire chain is activated, whereas in those supported by succinate (complex II substrate), complexes III–V are activated. Therefore, the fact that inhibition of state 3 respiration by Cd when glutamate-malate or succinate were used as substrates was equal to the inhibition attributed to active complex III alone suggests that Cd primarily impairs complex III. Similar to Cd, complex I-, II- and III-mediated state 3 respirations were inhibited by Ca albeit with much higher IC_{50} values and a different order of sensitivity: complex III > complex II > complex I. Complex IV-mediated state 3

TABLE 2
Dose Response of Cd and Ca Exposures, Singly and in Combination, on State 4 Respiration Rates (nmol O_2/mg protein/min) in Isolated Rainbow Trout Liver Mitochondria

		State 4 respiration (nmol O_2/mg protein/min)			
Exposure groups	Concentration (μM)	Complex I	Complex II	Complex III	Complex IV
Cd	Control	2.82 ± 0.10**	2.31 ± 0.10*	2.16 ± 0.03*	NM
	5	3.03 ± 0.22**	2.25 ± 0.11*	2.06 ± 0.19*	NM
	10	3.07 ± 0.28**	2.16 ± 0.21*	1.92 ± 0.16*	NM
	20	2.69 ± 0.28**	2.39 ± 0.10*	2.03 ± 0.14*	NM
	50	2.99 ± 0.19**	2.18 ± 0.02*	2.18 ± 0.14*	NM
Ca	Control	1.96 ± 0.36*	2.32 ± 0.56*	1.91 ± 0.13*	3.42 ± 0.32***
	5	1.80 ± 0.24*	2.04 ± 0.32*	2.17 ± 0.18*	3.42 ± 0.13***
	10	1.98 ± 0.35*	2.47 ± 0.39*	1.94 ± 0.18*	3.76 ± 0.16***
	20	1.78 ± 0.22*	2.06 ± 0.14*	2.18 ± 0.18*	3.71 ± 0.16***
	50	1.81 ± 0.18*	2.16 ± 0.21*	2.00 ± 0.15*	3.41 ± 0.10***
Cd + Ca	Control	2.19 ± 0.05*	2.47 ± 0.04*	1.91 ± 0.13*	NM
	5	2.02 ± 0.20*	2.48 ± 0.04*	1.73 ± 0.14*	NM
	10	1.90 ± 0.13*	2.63 ± 0.28*	2.02 ± 0.14*	NM
	20	2.07 ± 0.16*	2.52 ± 0.10*	1.986 ± 0.15*	NM
	50	1.97 ± 0.18*	2.33 ± 0.33*	1.98 ± 0.15*	NM

Notes. Complex IV-mediated state 4 respiration rates for Cd and Cd + Ca exposures were not measurable due to depletion of O_2 without transition to state 4. NM = not measured. Data are means ± SEM ($n = 5$). Values with different numbers of asterisks (*) are statistically different from one another (ANOVA, $p < 0.05$).

respiration was unaffected by Ca likely due to structural differences as explained for Cd. Collectively, we concluded that Cd- and Ca-induced dysfunctions in RTLM are, at least partly, a corollary of impairment of complexes I–III and that Cd is more potent than Ca.

We have consistently shown that Cd acts cooperatively with Ca to enhance mitochondrial dysfunction (Adiele *et al*., 2010, 2011, 2012). The present study demonstrates for the first time that the cooperative toxicity of Cd and Ca in RTLM is mediated, at least partly, through complexes I and III because the IC_{50} values for respiration driven by the two complexes were lower in the combined relative to single Ca and Cd exposures. The enhanced mitochondrial dysfunction could have resulted from additivity of direct toxic effects on the ETC

TABLE 3
Effect of Cd and Ca Exposures, Singly and in Combination, on RCR in Isolated Rainbow Trout Liver Mitochondria

		RCR			
Exposure groups	Concentration (μM)	Complex I	Complex II	Complex III	Complex IV
Cd	Control	7.53 ± 0.33****	7.00 ± 0.17****	7.91 ± 0.46****	NM
	5	5.77 ± 0.59**	7.49 ± 0.30****	8.16 ± 0.19****	NM
	10	5.46 ± 0.91**	6.46 ± 0.10***	7.16 ± 0.16****	NM
	20	5.21 ± 0.93**	6.35 ± 0.10***	5.55 ± 0.02**	NM
	50	5.10 ± 0.93**	5.79 ± 0.24**	4.76 ± 0.21**	NM
Ca	Control	7.55 ± 0.85****	7.46 ± 0.60****	7.91 ± 0.46****	3.21 ± 0.11*
	5	7.00 ± 1.02****	7.44 ± 0.24****	7.27 ± 0.50****	2.67 ± 0.21*
	10	6.86 ± 0.79****	6.27 ± 0.57***	5.82 ± 0.54***	2.90 ± 0.06*
	20	6.65 ± 0.38***	6.09 ± 0.91***	3.51 ± 0.00*	2.91 ± 0.09*
	50	5.93 ± 0.58***	5.12 ± 0.37**	3.21 ± 0.06*	3.26 ± 0.10*
Cd + Ca	Control	8.21 ± 0.53****	7.60 ± 0.33****	7.91 ± 0.46****	NM
	5	7.67 ± 0.73****	6.76 ± 1.01****	7.22 ± 0.41****	NM
	10	5.97 ± 0.78***	5.17 ± 0.59***	6.85 ± 0.43****	NM
	20	6.02 ± 1.07***	4.54 ± 0.94**	5.91 ± 1.10***	NM
	50	4.63 ± 0.26**	4.15 ± 0.81**	4.13 ± 0.78**	NM

Notes. Complex IV-mediated RCRs for Cd and Cd + Ca exposures were not measurable due to depletion of O_2 without transition to state 4. NM = not measured. Data are means ± SEM ($n = 5$). Values with different numbers of asterisks (*) are statistically different from one another (ANOVA, $p < 0.05$).

TABLE 4
Effect of Cd and Ca Exposures, Singly and in Combination, on ADP/O Ratio in Isolated Rainbow Trout Liver Mitochondria

		ADP/O ratio			
Exposure groups	Concentration (μM)	Complex I	Complex II	Complex III	Complex IV
Cd	Control	2.24 ± 0.14**	2.19 ± 0.19**	1.90 ± 0.17**	NM
	5	2.07 ± 0.13**	1.87 ± 0.13**	1.87 ± 0.17**	NM
	10	2.00 ± 0.09**	1.97 ± 0.06**	1.70 ± 0.08**	NM
	20	1.93 ± 0.17**	2.06 ± 0.26**	1.21 ± 0.11*	NM
	50	1.95 ± 0.03**	1.80 ± 0.18**	1.20 ± 0.04*	NM
Ca	Control	2.53 ± 0.13***	2.16 ± 0.07**	1.97 ± 0.17**	1.31 ± 0.08*
	5	2.19 ± 0.23**	2.08 ± 0.30**	1.97 ± 0.09**	1.22 ± 0.09*
	10	2.22 ± 0.05**	1.93 ± 0.16**	1.91 ± 0.15**	1.25 ± 0.07*
	20	2.30 ± 0.17**	1.93 ± 0.09**	2.17 ± 0.08**	1.16 ± 0.11*
	50	2.29 ± 0.09**	2.10 ± 0.18**	1.73 ± 0.21**	1.28 ± 0.06*
Cd + Ca	Control	2.51 ± 0.23**	2.17 ± 0.12**	2.28 ± 0.18**	NM
	5	2.81 ± 0.23***	2.49 ± 0.36**	2.17 ± 0.06**	NM
	10	2.65 ± 0.49**	1.80 ± 0.27**	2.14 ± 0.18**	NM
	20	2.26 ± 0.20**	2.13 ± 0.18**	2.03 ± 0.21**	NM
	50	1.72 ± 0.73**	1.13 ± 0.57*	1.92 ± 0.21**	NM

Note. Complex IV-mediated RCRs for Cd and Cd + Ca exposures were not measurable due to depletion of O_2 without transition to state 4. NM = not measured. Data are means ± SEM (n = 5). Values with different numbers of asterisks (*) are statistically different from one another (ANOVA, $p < 0.05$).

enzyme complexes with those on electron carriers and/or Krebs cycle. In this regard, Garceau *et al*. (2010) recently reported inhibition of citrate synthase by Cd in goldfish and, even more importantly, Ivanina *et al*. (2008) observed greater sensitivity of Krebs cycle enzymes to Cd than the other components of the substrate oxidation subsystem in eastern oyster mitochondria. A potential sequel of Krebs cycle inhibition is reduced generation of the coenzymes NADH and $FADH_2$ with diminished hydrogen delivery and electron transport. Together, the effects of Cd and/or Ca uncoupled RTLM as shown by the significant decrease in RCR and reduced the OXPHOS efficiency.

Measured ATP concentrations in phosphorylating mitochondria represent the net energy production taking into account unavoidable losses of ATP. The reduced ATP synthesis observed on exposure to Cd indicates impairment of the

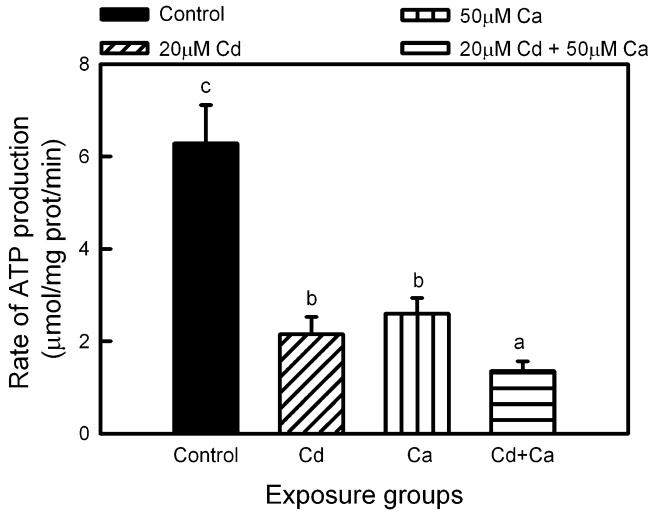

FIG. 4. Rate of ATP production in isolated RTLM fueled by malate-glutamate. The mitochondria were exposed to 20μM Cd and 50μM Ca singly and in combination. Data are means ± SEM ($n = 5$). Bars with dissimilar letters are significantly different from one another (ANOVA, $p < 0.05$).

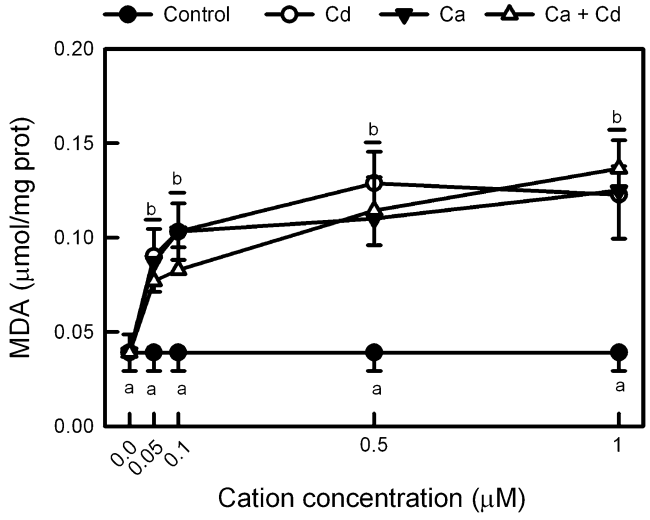

FIG. 5. MDA concentrations in isolated RTLM incubated for 1 h with 0 (control), 0.05, 0.1, 0.5, and 1μM Cd and Ca, singly and in respective equimolar combinations. Data are means ± SEM ($n = 5$). Points with dissimilar letters are significantly different from one another (ANOVA, $p < 0.05$).

mitochondrial phosphorylation and/or substrate oxidation subsystems and/or stimulation of the proton leak subsystem (Kesseler and Brand, 1994). Although our earlier study (Adiele *et al*., 2012) revealed that Cd impairs the ETC, the present study localizes the deleterious effects to ETC complexes I, II, and III and confirms the impairment of the substrate oxidation subsystem. Another possible cause of the observed ATP depletion is increased allocation of energy to the mitochondrial toxicant defense system. Specifically, counteracting the toxic effects of Cd/Ca exposure such as the oxidative stress (Fig. 5) likely required increased ATP consumption for maintenance/regeneration of manganese-dependent superoxide dismutase and the mitochondrial redox buffering systems constituted by glutathione, thioredoxin, and glutaredoxin (Marí *et al*., 2009). In agreement with our findings, Cd exposure decreased ATP content in rat liver mitochondria (Dorta *et al*., 2003), fetal rat cortical neurons (Lopez *et al*., 2003), marine diatom (Torres *et al*., 2000), and oyster hemocytes (Sokolova *et al*., 2004). Similar to Cd, elevated Ca exposure reduced ATP production with more severe effect when acting concurrently with Cd, supporting the theme of cooperative toxicity of these cations. Moreover, the finding that Ca exposure decreases ATP synthesis agrees with earlier studies in mice pancreatic B cells (Idahl and Lembert, 1995) and Ehrlich ascites tumor cells (Bogucka *et al*., 1995).

Elevated cellular levels of Cd and Ca have been associated with oxidative damage of macromolecules (Moradas-Ferreira *et al*., 1996; Turrens, 2003; Wang *et al*., 2004). In the present study, a product of oxidative damage of membrane lipid, MDA, was elevated on Cd and/or Ca exposures suggesting oxidative stress is a shared mechanism of toxicity of the two cations. Although the specific ROS and their sources were not identified, impairment of the ETC or impediment of electron flow enhances ROS production culminating in membrane lipid peroxidation and increased MDA levels (Li *et al*., 2003; Starkov *et al*., 2004; Turrens, 2003). Notably, impaired functions of complexes I and III promote ROS production (Brand, 2010; Reynafarje and Ferreira, 2008; Wang *et al*., 2004). For example, the impediment of electron flow through complex III results in accumulation of unstable semiubiquinone, which in turn donates lone electrons to O_2 with formation of $O_2^{\bullet -}$ (Li *et al*., 2003; Turrens, 2003). As well, stimulation of matrix dehydrogenases by Ca may lead to excessive electron transfer along the chain increasing ROS generation and predisposing mitochondrial membranes to oxidative damage (McCormack and Denton, 1990). These oxidative stress–mediated effects likely augmented the direct inhibition of ETC and uncoupling of OXPHOS by Cd and Ca thus contributing to the lower IC_{50} values in the Cd + Ca treatment in mitochondria fueled by complex I and III substrates.

In conclusion, our data suggest that Cd and Ca inhibit mitochondrial ETC complexes I, II, and III and induce oxidative stress thereby inhibiting OXPHOS and reducing ATP synthesis. Complex I-, II-, and III-mediated respirations were equally susceptible to Cd while the order of sensitivity to Ca was complex III > complex II > complex I. Interestingly, complex IV-driven respiration was resistant to both Cd and Ca, possibly due to its unique structure. The lower IC_{50} values for complexes I- and III-mediated respirations in the combined exposures persuasively indicate that the cooperative actions of Cd and Ca are mediated, at least in part, by these complexes. Although Cd and Ca showed qualitative similarities in toxicity, Cd is more potent than Ca as shown by the lower IC_{50} values for all the complexes and more severe deleterious effects on the mitochondria.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

Discovery Grant from the Natural Sciences and Engineering Research Council of Canada (RGPIN/311929).

ACKNOWLEDGMENTS

We are grateful to Cheryl Wartman and Jodie Bowmaster for technical support.

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