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INHIBITION OF CYTOCHROME OXIDASE BY DIBUCAINE

BRADLEY K. STRINGER and H. JAMES HARMON*
Department of Zoology, Oklahoma State University, Stillwater, OK 74078, U.S.A.

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Abstract—Dibucaine–HCl inhibited mitochondrial cytochrome *c* oxidase activity in intact mitochondria with 50% inhibition occurring at 1.1 mM dibucaine–HCl. Dibucaine–HCl did not prevent the reduction of cytochrome oxidase by ascorbate plus *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) when measured at 604 nm but prevented 50% of the absorbance change at 445 nm; dithionite reduced the oxidase completely. Dibucaine prevented binding of CO to oxidase reduced with ascorbate plus TMPD by preventing the reduction of cytochrome a_3. The midpotentials of cytochrome *c* and cytochrome oxidase, the visible absorbance wavelength maxima, and the position and intensity of the signals of the EPR spectrum of the oxidase were not affected. Dibucaine–HCl prevented ascorbate plus TMPD-driven reduction of the near infra-red detectable copper center associated with cytochrome *a*; dithionite subsequently reduced this center. Dibucaine–HCl inhibited cytochrome oxidase activity by interacting between cytochrome *a* and its associated copper. Since respiration was 8-fold less sensitive in submitochondrial particles, this site of inhibition is on the cytoplasmic side of the membrane.

Local anesthetics such as lidocaine, tetracaine, and dibucaine are used for anesthesia as well as in the treatment of cardiac arrhythmias. In the micromolar concentration range, the local anesthetics depress nerve action and cardiac signal conduction by blocking ion (sodium, potassium, calcium) channels in the membrane, either by interaction/perturbation with the ion channels themselves and/or the surrounding membrane lipids. Local anesthetics are known to interact with membrane lipids [1–3], but protein perturbation by, and interaction with, local anesthetics is also reported [4–7].

To increase our understanding of anesthetic–protein interactions, we have investigated the effects of dibucaine on the enzymatic activity and physicochemical characteristics of an integral membrane protein complex, cytochrome oxidase. While these studies do not attempt to explain the basis of anesthesia or antiarrhythmic action by interaction with specific ion channels, they may help explain the effects of general narcosis and mycocardial depression associated with administration of these drugs.

Inhibition of cytochrome *c* oxidase activity by local anesthetics has been described previously by others [8–12] as has the concentration dependence of the inhibition [8, 13]. Singer [8] and Casanovas *et al.* [11, 12] report that the molecule can interact electrostatically with the oxidase, possibly competing for the charged cytochrome *c* binding site to increase K_m. Because of a strong correlation between the concentration dependence and the octanol/water coefficient for many anesthetics including dibucaine, Singer [8, 9] and Casanovas *et al.* [11] suggested a hydrophobic interaction, most likely with the lipid associated with the oxidase [13]. Chazotte and Vanderkooi [14] and Vanderkooi and Chazotte [15] demonstrated that the concentration of dibucaine needed to inhibit cytochrome oxidase in rat liver submitochondrial membranes is inversely related to temperature, further suggesting a hydrophobic interaction. They suggested a reversible perturbation of cytochrome oxidase protein conformation but could not determine if the anesthetics react with the protein directly (as suggested by their observation of inhibition of lipid-free isolated mitochondrial ATPase [16–19]) or with its "boundary lipid" [13].

Despite these extensive studies on the mechanism and type of inhibition, the site of action of dibucaine and other anesthetics in cytochrome oxidase has not been identified. In this report, we define the functional site of dibucaine inhibition in oxidation and reduction of cytochrome oxidase and suggest its topographical location in the membrane.

MATERIALS AND METHODS

Mitochondria containing endogenous cytochrome *c* were isolated from fresh beef heart by the method of Crane *et al.* [20]. Cytochrome *c*-depleted beef heart mitochondria were isolated as described by Harmon and Crane [21]. Exogenous cytochrome *c* (Sigma, Type VI) was added to the mitochondria (100 μg cytochrome *c*/mg mitochondrial protein) prior to recording spectra. Differences in spectra or kinetic activity were not observed between these two mitochondrial preparations.

Cytochrome *c* oxidase and succinate oxidase activities were measured polarigraphically at 25° in a 1.7-mL volume water-jacketed glass chamber fitted with a Clark oxygen electrode in medium containing 83 mM sodium phosphate buffer (pH 7.4) using 100 μg mitochondrial protein and 200 μg cytochrome *c* (Sigma type VI) in each assay. Sodium ascorbate (9 mM) and 28.1 μM (final concentrations) *N,N,N′,N′*-tetramethyl-*p*-pheneylenediamine dihydrochloride (TMPD†) were used as substrate to

* To whom correspondence should be addressed.

† Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride; CuA, infra-red detectable copper center associated with cytochrome *a*; PMS, phenazine methosulfate; and DAD, diaminodurene; 2,3,5,6-tetramethyl-*p*-phenylenediamine.

measure cytochrome oxidase activity. Non-enzymatic auto-oxidation of the substrates was determined following the addition of 400 μg of neutralized protamine sulfate to inhibit the oxidase; this rate was subtracted from the overall rate to yield cytochrome oxidase-dependent oxygen consumption. Sodium succinate (27.6 mM final concentration) was used as substrate for succinate oxidase activity.

Lipid-depleted purified cytochrome oxidase used for EPR spectra was isolated from beef heart mitochondria by the procedure of Yu *et al.* [22].

Dual wavelength kinetics using the 445 *minus* 465 nm or 604 *minus* 630 nm wavelength pairs were recorded at room temperature with a Johnson Research Foundation (University of Pennsylvania) DBS-3 scanning dual wavelength spectrophotometer. Spectra in the near infra-red region were recorded with the same instrument with a Kodak Wratten No. 15 filter used as a second order filter. Spectra of carboxy-oxidase were recorded at room temperature using a Varian DMS-100S spectrophotometer modified to function in dual wavelength mode. Ascorbate (5 mM final concentration) plus 28.1 μM TMPD or dithionite was used as reductants as noted in the figure legends.

Beef heart mitochondria used in these experiments were suspended at 2 mg protein/mL in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4). Carboxy-oxidase (room temperature spectra) was generated by bubbling the ascorbate plus TMPD-reduced sample with 100% CO for 5 min.

The oxidation–reduction potentials of cytochrome oxidase and cytochrome *c* were measured at room temperature with a platinum-calomel electrode pair as described by Dutton and Wilson [23] using the DBS-3 dual wavelength instrument as described previously [24]. 2,3,5,6-Tetramethyl-*p*-phenylenediamine (DAD, 60 μM), 156 μM phenazine methosulfate (PMS), and 3 μM duroquinone (final concentrations) were used as potential mediators. The potential of the solution was varied by addition of ferricyanide and/or dithionite solutions.

Electron paramagnetic resonance spectra were recorded at 8–10°K with an ER 2000 series Bruker spectrometer equipped with an Oxford Instruments helium transfer/dewar system. Samples contained approximately 6 mg purified oxidase/mL in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4). Spectra were recorded at approximately 9.49 GHz with 6.3 mW power, 100 KHz modulation frequency, and 4 G modulation.

RESULTS

The inhibition of ascorbate plus TMPD-driven oxygen consumption by cytochrome oxidase in intact beef heart mitochondria was dependent on the concentration of dibucaine–HCl present (Fig. 1). In the presence of 1.1 mM dibucaine, 50% inhibition was observed with over 85% inhibition obtained with 2 mM dibucaine. Respiration was inhibited 70% at 1.5 mM dibucaine, the concentration used in subsequent spectrophotometric measurements in this report. Complete restoration of activity following washing of 1.5 mM dibucaine-inhibited mitochondria with bovine serum albumin indicated that the effects were reversible upon removal of the drug.

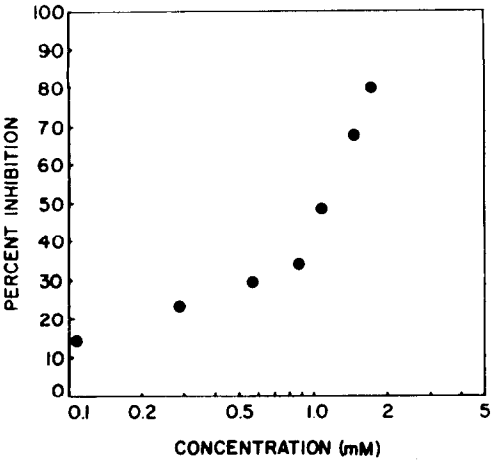

Fig. 1. Concentration dependence of the inhibition of cytochrome oxidase activity (oxygen consumption) by dibucaine–HCl in intact beef heart mitochondria at 25°. Control (uninhibited) activity: 592 ng-atom O/min/mg protein.

Addition of ascorbate plus TMPD to mitochondria in the presence or absence of 1.5 mM dibucaine–HCl resulted in approximately 90% reduction of cytochrome oxidase (compared to the dithionite-reduced sample) as measured by the absorbance increase at 604 nm (cf. Fig. 2, A and B). The absorbance at 445 nm upon reduction of the oxidase decreased in the presence of 1.5 mM dibucaine when ascorbate plus TMPD was used as reductant (in the absence of the drug, 90% reduction was observed as shown in Fig. 2, C and D). Addition of dithionite caused a 100% increase in the 445 nm absorbance in the presence of dibucaine; dithionite is a strong non-physiological reductant with a midpotential of approximately −436 mV at pH 7.4 and is capable of reducing the hemes of cytochrome oxidase directly, whereas ascorbate cannot reduce the oxidase in the absence of cytochrome *c*. Because of its low midpotential, dithionite is capable of reducing all components of the respiratory chain.

The extent of reduction of cytochrome oxidase as measured by the increase in 445 nm absorbance following the addition of ascorbate (relative to the total reduction with dithionite) was dependent on the concentration of dibucaine–HCl present, as shown in Fig. 3. In the absence of dibucaine, addition of ascorbate plus TMPD caused an increase in absorbance to a level 90% of that observed in the presence of dithionite. The extent of 445 nm absorbance decreased a maximum of 50% at 1.5 mM and did not decrease further in the presence of higher concentrations of drug. Subsequent experiments were performed at 1.5 mM or higher concentrations where the maximal decrease in 445 nm absorbance and maximal inhibition of electron transfer from cytochrome *a* to cytochrome a_3 occurred.

The "neoclassical" model for cytochrome oxidase holds that approximately 80–90% of the absorbance of reduced oxidase at 604 nm is due to ferrous cytochrome *a*, and the remainder due to ferrous cyto-

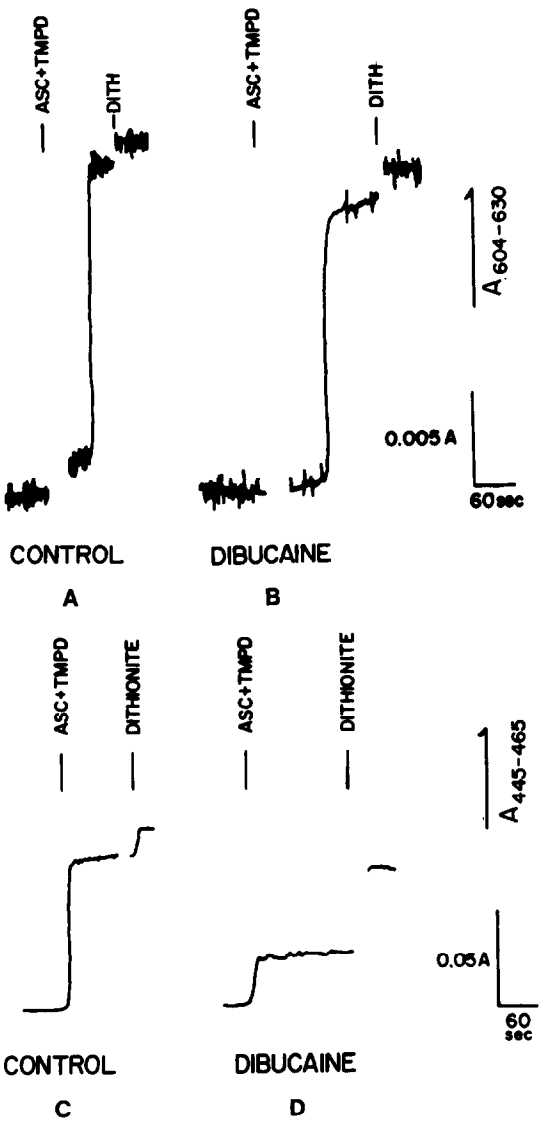

Fig. 2. Reduction of cytochrome oxidase as measured using the 604 *minus* 630 nm pair in the absence (A) and presence (B) of 1.5 mM dibucaine–HCl at room temperature and the reduction of cytochrome oxidase as measured using the 445 *minus* 465 nm pair in the absence (C) and presence (D) of 1.5 mM dibucaine–HCl. Cytochrome *c*-depleted mitochondria were suspended at 2 mg/mL in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4). Ascorbate (5 mM) and TMPD (28.1 μM) with 200 μg cytochrome *c* (Sigma, type VI) were used as reductants.

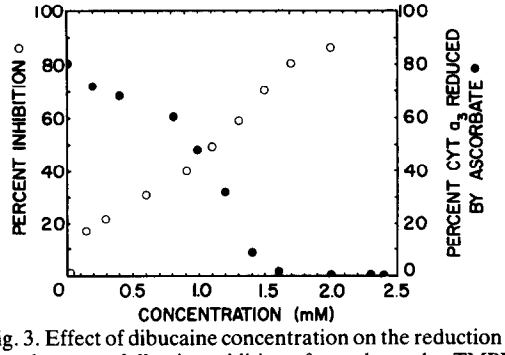

Fig. 3. Effect of dibucaine concentration on the reduction of cytochrome a_3 following addition of ascorbate plus TMPD. Conditions were as described in the legend of Fig. 2. Total reduction of cytochrome oxidase and maximal absorbance increase was measured after the addition of dithionite. Control (uninhibited) activity: 592 ng-atom O/min/mg protein. Full scale absorbance change was 0.05 A, corresponding to 2.1×10^{-9} moles cytochrome *a*.

chrome a_3; both cytochromes absorb light equally at 445 nm [25]. The data in Figs. 2 and 3 are consistent with this model. Over 80% of the 604 nm and approximately 50% of the 445 nm absorbance were recorded in the presence of the anesthetic when reduced with ascorbate plus TMPD. Addition of dithionite caused only a slight increase in 445 nm absorbance. In the absence of the anesthetic, essentially complete reduction of both cytochromes was observed. Thus, dibucaine–HCl inhibited electron transport between cytochromes *a* and a_3, allowing the reduction of cytochrome *a* (80% of 604 nm and 50% of 445 nm absorbance) but not cytochrome a_3.

The presence of dibucaine did not alter the midpotential of cytochrome *c* (Harmon HJ, unpublished results). The midpotential of cytochrome oxidase was increased from 234 ± 4 to 255 ± 4 mV in intact mitochondria in the presence of 1.5 mM dibucaine–HCl.

In the presence of up to 10 mM dibucaine–HCl, neither the position nor the intensity of the g = 3 EPR signal due to ferric cytochrome *a* or the g = 2 signal due to CuA was altered. The field position of the signal in the presence of dibucaine was within 5 G that of the untreated enzyme while the half-bandwidth of the g = 3 signal in both cases was 53 G.

As shown in Fig. 4, ascorbate plus TMPD was capable of reducing the "visible" CuA moiety in the absence but not the presence of either 1.5 or 2 mM dibucaine–HCl. Reduction of the CuA center is shown by a decrease in absorbance in the 840–860 nm region of a reduced minus oxidized difference spectrum [26, 27]. In the presence of dibucaine, addition of dithionite caused a decrease in near infra-red absorbance, indicating that the CuA center is capable of being reduced by a strong reductant. Similar results have been obtained using either isolated oxidase or mitochondria washed with bathophenanthroline sulfate and bathocuproine sulfonate to remove adventitious copper [26–29].

Reduced cytochrome oxidase exhibited a characteristic alpha band at 604 nm; addition of CO resulted in an increase in 590 nm absorbance due to formation of ferrous carboxy-cytochrome a_3. In the presence of dibucaine, the alpha band at 604 nm was still observed with ascorbate plus TMPD as reductant but the absorbance increase at 590 nm following CO addition was not observed; the 590 nm carboxy-oxidase band was observed when dithionite was added to the sample, however (data not shown). This is to be expected since dibucaine prevented reduction of cytochrome a_3 by ascorbate plus TMPD.

DISCUSSION

Chazotte and Vanderkooi [14] reported LD_{50}

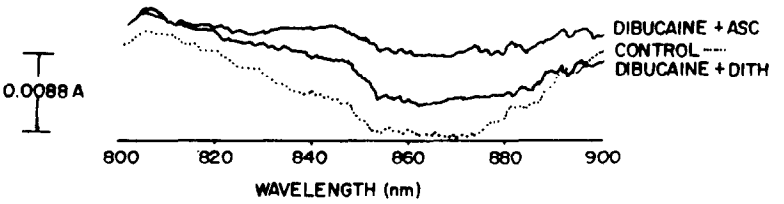

Fig. 4. Effect of dibucaine–HCl on the reduction of the IR-detectable CuA center in intact mitochondria using ascorbate plus TMPD as reductant. Cytochrome *c*-containing mitochondria were suspended at 4 mg/mL in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4). The data represent the signal averaged from sixteen separate accumulations of spectra. The spectra were recorded at room temperature.

values for dibucaine of 9 mM for cytochrome oxidase and 1.5 mM for succinate oxidase activities in submitochondrial particles. Singer [8] reported 50% inhibition of cytochrome oxidase activity at a 5 mM concentration. The data presented in Fig. 1 illustrate that 50% inhibition of cytochrome oxidase activity in intact mitochondria occurred at 1.1 mM dibucaine–HCl. When succinate oxidase activity was measured, 50% inhibition of activity was observed at 1.25 mM in intact mitochondria and at 9 mM dibucaine–HCl in 94% inverted submitochondrial particles isolated by the procedure of Harmon [30]. The concentration dependence of the inhibition by dibucaine–HCl (water soluble and apparently membrane impermeant) was dependent on the orientation of the membrane vesicles used and indicates that the inhibitory site is present on the C side of the membrane and not on the M side.

The midpotentials of cytochrome *c*, cytochrome *a*, and cytochrome a_3 were not affected by the presence of dibucaine–HCl, indicating that these centers are not altered directly by the drug. The wavelength maxima of the cytochromes were not altered; the wavelength maximum and extinction coefficients of cytochrome *c* (data not shown) also were not altered by the drug. The lack of change in the EPR spectrum and the small change in cytochrome oxidase midpotential indicate that the site of action of the drug is not cytochrome *a*. The lack of change in the characteristics of carboxy oxidase suggests that the anesthetic does not alter the ligand-binding capability of cytochrome a_3 and that cytochrome a_3 is not affected by dibucaine.

The perturbation of the EPR spectrum of CuA by dysprosium compounds [31] indicates that the CuA center is on the C side of the membrane. Our data are in agreement with this conclusion since dibucaine–HCl acted at the CuA center and activity in intact mitochondria was more sensitive to the drug that activity in inverted particles; the dibucaine-sensitive site is on the cytoplasmic side.

Dibucaine did not act directly at cytochrome *a* or a_3, based on its inability to alter their physico–chemical characteristics. In this way it is much different from the conventional oxidase inhibitors such as CO, sulfide, cyanide, azide, or fluoride but is similar to hydrophobic metal chelators such as bathophenanthroline or bathocuproine which are believed to act at the coppers of cytochrome oxidase [29].

The action of dibucaine is similar to that of the inhalational anesthetic nitrous oxide in that it, too, does not act as a ligand to or oxygen donor for cytochrome oxidase. Nitrous oxide has been shown to not alter the CO binding to the oxidase (from IR spectra) or the Soret absorbances of the oxidase in either the oxidized or reduced state. Einarsdottir and Caughey [32] were able to demonstrate inhibition of electron transfer from cytochrome *c* to the oxidase but did not define the site of inhibition further. Nitrous oxide was shown to exist in both lipid (one IR resonance band) and protein environments (responsible for three IR bands). From our findings it is not possible to determine if the hydrophobic dibucaine acts by perturbing the lipid or protein components (or both) of the oxidase. Since only one site of inhibition was observed (at the visible copper), dibucaine likely interacts with the protein component; one might expect alteration of the cytochromes if dibucaine were to alter lipid–protein interactions. These experiments cannot exclude the possibility that multiple dibucaine molecules are involved in the effects reported here. That dibucaine inhibits oxygen consumption in lipid-depleted isolated oxidase as well (data not shown) strongly suggests interaction directly with protein components.

Dibucaine will be useful as a tool to allow the study of the physico–chemical properties of cytochromes *a* or a_3 without interference (optical absorbance or potential) from the other cytochrome. While $a^{3+}\ a_3{}^{2+}$ mixed valence oxidase can easily be made [33–35], dibucaine now allows the generation of the corresponding $a^{2+}\ a_3{}^{3+}$ mixed valence form of the oxidase.

We propose that dibucaine may be useful in elucidating the role and sequence of the oxidation of cytochrome *a* in the formation of oxygen intermediates that form during the reduction of oxygen [33, 34]. Further, blockage of electron transport from cytochrome *a* will allow more accurate measurement of the rates of electron transfer between the coppers and cytochrome a_3 [36].

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