Biochemical Pharmacology, Vol. 29, pp. 2651–2655.

0006-2952/80/1001–2651 $02.00/0

INTERACTION OF DRUGS WITH A MODEL MEMBRANE PROTEIN

EFFECTS OF FOUR LOCAL ANESTHETICS ON CYTOCHROME OXIDASE ACTIVITY

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(*Received* 5 *February* 1980; *accepted* 24 *April* 1980)

Abstract—Cytochrome oxidase is a mitochondrial membrane protein which catalyzes the oxidation of ferrocytochrome *c* and transfers the electrons to molecular oxygen. When tested in low ionic strength buffer, the enzyme displayed both high and low affinity binding sites for the substrate, cytochrome *c*, with binding being chiefly via electrostatic forces. Benzocaine, a non-charged local anesthetic, had no effect on oxidase activity. The cationic local anesthetics dibucaine, tetracaine and procaine caused a mixed-type inhibition of the low affinity site with the relative order of potency being dibucaine > tetracaine >procaine. The former two also inhibited the high affinity site. It is postulated that the charged end of the anesthetic competes with substrate for binding, while the non-polar end interacts with oxidase-associated phospholipids to produce non-productive complexes.

Many drugs are either known or believed to act through an effect on particular cellular membranes. Structurally, biological membranes consist of a phospholipid bilayer matrix and a number of associated protein molecules that are responsible for the more sophisticated properties of the membrane. In previous work we studied drug:lipid interactions exclusively, using a model bilayer (liposome) system [1–3]. This same system can be extended by incorporating proteins into the bilayer, thus permitting the study of functional interactions between protein and lipid and the effects of drugs on these interactions. Ideally, the protein molecule(s) that constitutes the target for a given set of drugs would be used. However, with very few exceptions, these proteins have either not been or cannot be extracted in pure enough form for studies in model systems. One such exception is the protein cytochrome oxidase. This molecule is a well-defined mitochondrial integral membrane protein with a known biological function: the vectorial transfer of electrons from cytochrome *c* to molecular oxygen [4]. More importantly, in terms of future studies, it can be functionally incorporated into model lipid bilayers [5]. For these reasons cytochrome oxidase was chosen as a suitable model membrane protein.

Our first objective, and the purpose of this report, is to describe the effects of specific drugs on the function of the isolated protein. Since cytochrome *c* (the substrate) is positively charged at physiological pH and since its binding to the oxidase is predominantly electrostatic [4, 6], we chose a series of cationic local anesthetics as a suitable set of drugs to test.

MATERIALS AND METHODS

Materials. Cytochrome *c* (type V1 from horse heart), sodium ascorbate, sodium cholate and TMPD (N,N,N^1,N^1-tetramethyl *p*-phenylene, diamine dihydrochloride) were obtained from the Sigma Chemical Co., St. Louis, MO. The four local anesthetics were purchased from ICN Pharmaceuticals, Plainview, NY. All other chemicals were of reagent grade wherever possible.

Cytochrome oxidase. Cytochrome oxidase was extracted from beef heart and purified according to the method of Kuboyama *et al.* [7]. The final preparation was dissolved in 100 mM phosphate buffer–0.25 per cent Tween 80 (pH 7.4) and stored at −80°. Cytochrome aa_3 concentration was measured spectrophotometrically using a millimolar extinction coefficient of 24 at 605 nm (reduced–oxidized) [8]. Protein concentration was measured using the biuret reaction [9]. The oxidase preparations obtained had heme to protein ratios of 7 to 8 nmoles heme *a* per mg protein.

Polarographic assay of oxidase activity. Rates of oxygen uptake were measured with a Yellow Springs Instruments oxygen electrode fitted to a conventional chart recorder. The assay mixture had a volume of 3 ml and contained cytochrome *c* (concentration range indicated in the figures), 6 mM sodium ascorbate, 0.18 mM TMPD, and 1 per cent Tween 80, all in phosphate buffer (20 or 67 mM) of pH 6.5. After a stable baseline was achieved, cytochrome oxidase (final concentration 0.022 μM) was added. Autooxidation (oxygen uptake in the absence of enzyme) was found to vary from day to day but could be minimized by preparing fresh reagents daily. Measurements were made at 30° and at this temperature the oxygen concentration of air-saturated buffer was assumed to be 200 nmoles/ml [10].

Because different enzyme preparations were used over the course of these experiments, a separate series of controls was run prior to the use of one of the local anesthetics. The anesthetic was added to the assay mixture prior to the addition of the enzyme.

Enzyme activity is expressed in terms of a turnover number (TN) that is calculated by multiplying the oxygen consumption by four* and dividing by the oxidase concentration. The maximum turnover number (TN_{max}) is the value extrapolated to infinite cytochrome *c* concentration.

Preliminary experiments indicated that the enzyme displayed maximum activity at a pH of about 6.5, as has been reported in the literature [4]. All subsequent experiments were performed at this pH. In addition, since several anions can effect the kinetics of the cytochrome oxidase catalyzed reaction, a single buffer (phosphate) was used for all experiments [11].

RESULTS

Figure 1 illustrates the effects of four local anesthetics on oxidase activity. These experiments were done in 67 mM phosphate buffer. The data could be fitted to simple Michaelis–Menten kinetics, and in Fig. 1 the results are presented in the form of Lineweaver–Burk plots. Benzocaine, the one uncharged anesthetic, had no detectable effect on enzyme function. The effects of the three other anesthetics (procaine, tetracaine and dibucaine) are most consistent with a mixed-type of inhibition for they cause both a decrease in maximum enzyme turnover number (TN_{max}) and an increase in the K_m. Table 1 summarizes the kinetic parameters obtained in these experiments.

A mixed type of inhibition can arise from several situations [12]. The simplest scheme is one in which the enzyme–inhibitor complex has a lower affinity than the free enzyme for the substrate and the enzyme–substrate–inhibitor (ESI) complex is nonproductive or dead-end. More complicated schemes occur when the ESI complex is only partially nonproductive. The distinctions between these various models, however, are difficult† and hence we will assume the first model is operative with respect to these local anesthetics. With this model the dissociation constant for the enzyme–inhibitor complex (K_i) can be determined by a plot of the reciprocal slopes of Fig. 1 versus the corresponding concentration of inhibitor (local anesthetic) [12]. This plot is illustrated in Fig. 2 and yields K_i values of 2 mM for dibucaine, 3.2 mM for tetracaine, and 12 mM for procaine. Furthermore, the plot appears to be linear, which is also evidence in favor of the simple scheme assumed above [12].

The experiments illustrated in Fig. 1 were performed in 67 mM phosphate buffer. The K_m and TN_{max} of the uninhibited enzyme (Table 1) are consistent with what has been described in the literature as the low affinity kinetic phase. A high affinity kinetic phase, however, has also been described,

* Four moles of cytochrome *c* are oxidized for each mole of oxygen consumed.

† Multiple inhibitor concentrations must be used to distinguish between these models. Due to solubility and ionic strength effects, anesthetic concentrations higher than 10 mM could not be used.

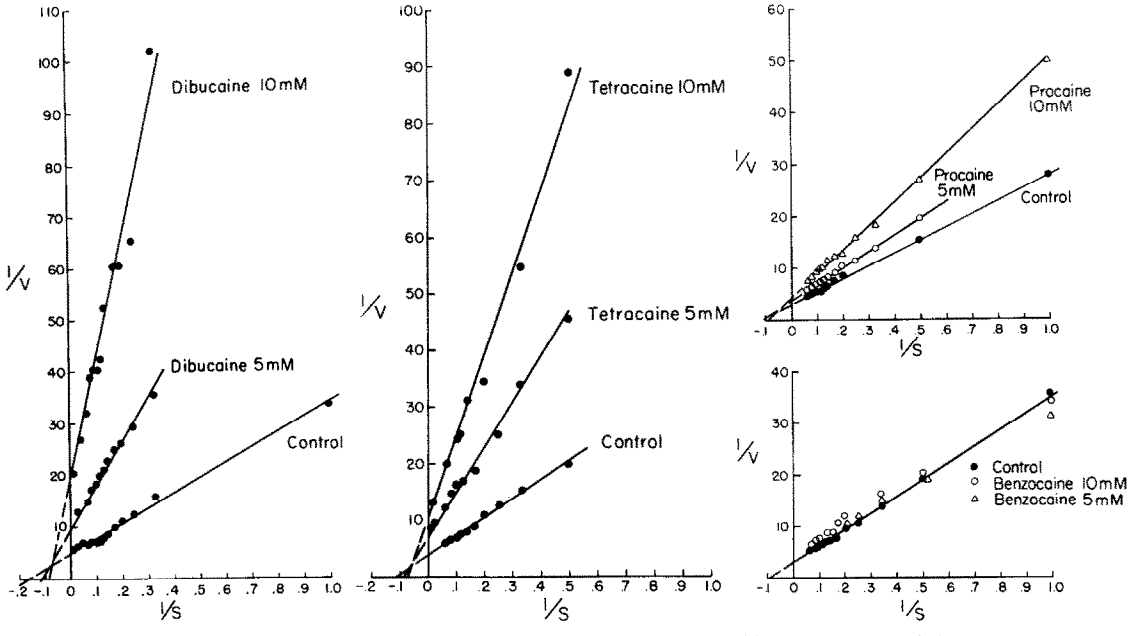

Fig. 1. Activity of cytochrome oxidase in the presence and absence of local anesthetics (Lineweaver–Burk plot). Enzyme activity was measured as described in the text. The final assay mixture had a volume of 3 ml and contained 1–100 μM cytochrome *c*, 6 mM, sodium ascorbate, 0.18 mM TMPD, 0.022 μM cytochrome oxidase, and 1 per cent Tween 80, all in 67 mM phosphate buffer, pH 6.5. The ordinate ($1/v$) is the reciprocal of the turnover number (sec^{-1}) multiplied by 10^3. The abscissa ($1/s$) is the reciprocal of the cytochrome *c* concentration (μM). Anesthetic agents were present at a concentration of 5 or 10 mM as indicated. Lines were drawn by the method of least squares. As a control for changes in ionic strength due to addition of the anesthetic, oxidase activity was compared in 77 mM and 67 mM buffer. No significant difference in enzyme activity was observed (data not shown).

Table 1. Kinetic parameters*

(A) 67 mM Phosphate buffer

	K_m (μM)	TN_{max} (sec^{-1})
Control	5.9	200
Dibucaine (5 mM)	8.3	100
Dibucaine (10 mM)	11.1	50
Control	8.3	250
Tetracaine (5 mM)	11.1	145
Tetracaine (10 mM)	14.3	100
Control	7.8	300
Procaine (5 mM)	8.9	275
Procaine (10 mM)	10.9	230
Control	10	300
Benzocaine (10 mM)	10	300

(B) 20 mM Phosphate buffer

	High affinity phase		Low affinity phase	
	K_m (μM)	TN_{max} (sec^{-1})	K_m (μM)	TN_{max} (sec^{-1})
Control	0.1	42	1.5	150
Dibucaine (10 mM)	0.32	32	5.6	100
Procaine (10 mM)			2.0	140

(C) Dissociation constants of anesthetic–enzyme complexes

Anesthetic	Low affinity phase K_i (mM)	High affinity phase K_i (mM)
Dibucaine	2 (Fig. 2) 2.1 (Fig. 3)	3.0 (Fig. 3)
Tetracaine	3.2 (Fig. 2)	
Procaine	12 (Fig. 2) 23 (Fig. 3)	

* The kinetic parameters in sections A and B were derived from Figs. 1 and 3, respectively. The K_i values in section C were obtained from Figs. 2 and 3 as indicated.

although, as well illustrated in Refs. 13 and 14, it only becomes apparent when low ionic strength buffer is used.* Figure 3 illustrates the effects of dibucaine, procaine and benzocaine on oxidase function, measured at pH 6.5 in 20 mM phosphate buffer. The results are presented as an Eadie–Scatchard plot because this type of graph depicts deviations from linearity more clearly than a plot of $1/v$ versus $1/s$. The untreated oxidase clearly yields a biphasic kinetic pattern.

Benzocaine and procaine had no significant effects on the high affinity phase† but the latter did cause a mixed type of inhibition of the low affinity phase. Dibucaine caused a mixed type of inhibition of both phases. Although not illustrated, tetracaine (10 mM)

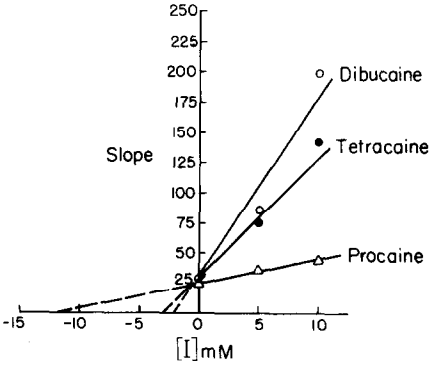

Fig. 2. Slope replot of Fig. 1. The slopes of the lines from the reciprocal plots of Fig. 1 are on the ordinate. The millimolar anesthetic concentrations are on the abscissa. The intercept of a given line on the × axis gives K_i.

* When low substrate concentrations (<1 μM) were used in 67 mM phosphate buffer, a biphasic kinetic plot was not obtained, consistent with the data in Refs. 13 and 14.

† One might argue that procaine caused a reduction in the TN_{max} of the high affinity phase. However, the turnover numbers in the presence of very low substrate concentrations are quite small and subject to considerable variation. Hence it is difficult to assess how much significance to attach to the difference between the procaine high affinity phase and the high affinity phase of the unhibited enzyme. Certainly the contrast between dibucaine and procaine on this phase is quite striking.

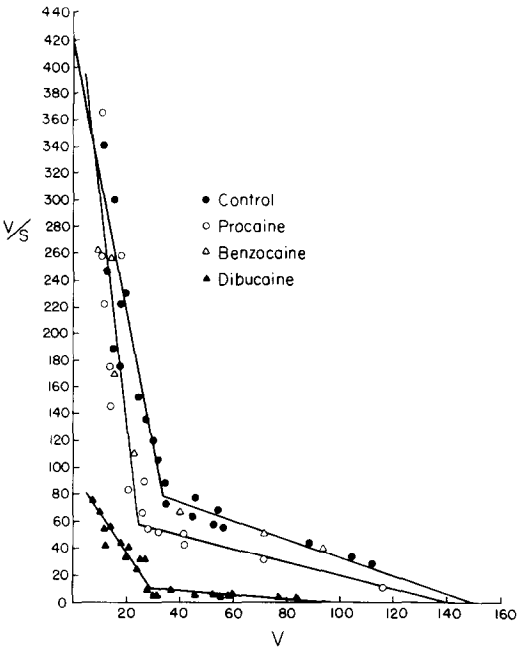

Fig. 3. Effects of dibucaine (10 mM), procaine (10 mM) and benzocaine (10 mM) on cytochrome oxidase activity (Eadie–Scatchard plot). Enzyme activity was measured as described in the text. The final assay mixture was similar to that described in the legend to Fig. 1 except that the buffer used was 20 mM phosphate (pH 6.5) and the cytochrome *c* concentration ranged from 0.03 to 10 μM. The ordinate (v/s) is the enzyme turnover number (sec^{-1}) divided by the substrate concentration (μM). The abscissa is the enzyme turnover number (sec^{-1}). As a control for changes in ionic strength due to addition of anesthetic, oxidase activity was compared in 20 mM and 30 mM buffer. No significant difference in enzyme activity was observed (data not shown). Lines were drawn by the method of least squares. Tetracaine at 10 mM gave results indistinguishable from dibucaine.

in this low ionic strength buffer gave results indistinguishable from dibucaine.

DISCUSSION

Cytochrome oxidase catalyzes the oxidation of ferrocytochrome *c* with the transfer of electrons from the substrate to molecular oxygen. It has been demonstrated that the overall rate of reaction is independent of the oxygen concentration under the usual aerobic assay conditions [15]. Margoliash and coworkers [13, 14, 16] compared the kinetics of cytochrome *c* oxidation with the measured binding of the substrate to the oxidase. They observed that the steady-state kinetics are dominated by the binding parameters and that the Michaelis constants (K_m values) obtained are a good measure of the actual dissociation constants for cytochrome *c*. The following description of the steps involved in cytochrome *c* binding to the oxidase is taken from Refs. 14 and 16.

Cytochrome oxidase exists as a dimer with two cytochrome *c* binding sites per protomer. The first molecule to bind to the enzyme is responsible for the high affinity kinetic phase. The binding process predominantly involves electrostatic interactions between a positively charged domain on the cytochrome *c* molecule and a negatively charged domain on the enzyme. This 1:1 cytochrome *c*–oxidase complex is capable of binding a second molecule of cytochrome *c* in a catalytically active manner, accounting for the lower affinity kinetic phase. The presence of the first positively charged molecule of cytochrome *c* reduces the affinity of the second.

The low affinity site measured in 67 mM phosphate buffer had a TN_{max} varying from 200 to 300 sec^{-1} and a K_m ranging from 5.9 to 10 μM (Table 1). This same phase measured in 20 mM phosphate buffer had a TN_{max} of 150 sec^{-1} and a K_m of 1.5 μM. The reason for this difference is not clear but may represent the effect of changes in ionic strength alone.* The high affinity phase had a K_m about 15-fold less and a TN_{max} approximately one quarter that of the low affinity phase. That is, the first cytochrome *c* molecule binds more strongly and turns over more slowly than the second molecule.

A comparison of the effects of the four local anesthetics underscores the importance of electrostatic interactions for the anesthetic-induced reduction in oxidase activity. Benzocaine, an electrically neutral molecule, has no effect on enzyme activity. The three charged anesthetics reduce the affinity of the oxidase for cytochrome *c*, and cytochrome *c* binds largely via electrostatic forces. It seems reasonable, therefore, to assume that the charge–charge interaction between anesthetic and enzyme involves the "c" binding site or at least an area close to it. Charge–charge interactions alone, however, do not completely explain the anesthetic effects. Procaine, dibucaine and tetracaine have similar pK values [17] and hence on a charge basis should be equipotent in inhibiting oxidase function. Such is not the case. In terms of the low affinity site, the relative order of effectiveness is dibucaine > tetracaine > procaine (Fig. 2 and Table 1). In terms of the high affinity site, dibucaine is much more potent an inhibitor than procaine, with tetracaine giving results similar to dibucaine. This sequence, dibucaine > tetracaine > procaine, is identical to that of their relative anesthetic potencies measured, for example, by the concentrations of these agents required to cause a 50 per cent reduction in the height of the action potential of a frog sciatic nerve (Table 2).

The additional inhibitory effects of these anesthetics (i.e. those not due to charge–charge interactions) most likely result from hydrophobic interactions with either the protein itself or with oxidase-associated phospholipids. Cytochrome oxidase preparations do contain about 20 per cent, w/w, of phospholipid to protein. This phospholipid compnent is critical to oxidase activity, for delipidated enzyme is inactive [20]. It is believed that these phospholipids are necessary to accelerate the internal transfer of electrons between the two heme moieties (a, a_3) within the oxidase complex [20]. Although

* The low affinity phases measured in 67 mM and 20 mM phosphate buffer however, do differ in one other respect. The cationic anesthetics were more potent inhibitors of TN_{max} when the 67 mM buffer was used (Table 1).

Table 2. Comparison of the dissociation constants for the enzyme–anesthetic complex with the anesthetic concentrations causing disruption of phospholipid organization and nerve blockade*

K_i (low affinity site)* (mM)	Anesthetic concentration causing a 50 per cent reduction in the height of the frog sciatic nerve action potential† (mM)	Anesthetic concentration required to double the width of transition endotherm of dipalmitoyl phosphatidylcholine liposomes‡ (mM)
Dibucaine 2(2, 2.1)	0.2	1.8
Tetracaine 3.2	0.6	3.3
Procaine 17.5 (12, 23)	8.0	17

* The K_i values for the low affinity site are taken from Table 1.

†. The data for the frog sciatic nerve preparation come from Ref. 18. These experiments were performed at 20° and at pH 7.2.

‡ The anesthetic data for dipalmitoyl phosphatidylcholine liposomes are from Ref. 19.

the experiments reported in this paper did not discriminate between protein and lipid as the site of such hydrophobic interactions, several observations suggest that the anesthetics most probably interact with the phospholipids. First, the sequence dibucaine > tetracaine > procaine is identical to the relative strengths of their hydrophobic interactions with isolated phospholipids as measured by n.m.r. spectroscopy [19]. Second, the anesthetic–enzyme K_i values for the low affinity site are very similar to the anesthetic concentrations that cause a marked disruption of phospholipid organization measured by a 50 per cent decrease in the phase transition cooperativity of dipalmitoyl phosphatidylcholine liposomes (Table 2) [21[.

In summary, cationic local anesthetics cause a mixed-type inhibition of cytochrome oxidase that appears to depend on both electrostatic and nonpolar interactions of anesthetic with enzyme. One working model to account for these observations is as follows. The charged portion of the anesthetic would compete with the substrate cytochrome *c* for binding, while hydrophobic interactions between the anesthetic and oxidase-associated phospholipids would result in non-productive or dead-end substrate–enzyme complexes.

Acknowledgements—I would like to thank Mrs. M. Dinda for expert technical assistance and Dr Peter Nicholls for help with the extraction of cytochrome oxidase. This research was supported by the Medical Research Council of Canada.

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