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Local Anesthetics: A New Class of Partial Inhibitors of Mitochondrial ATPase

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Abstract

The following characteristics are reported for mitochondrial ATPase prepared by the chloroform extraction method: (1) The pH optimum for enzyme activity is at 8.0. (2) The neutral anesthetic benzocaine inhibits the enzyme at all pH values. (3) Reciprocal plots of $1/v$ versus $1/[ATP]$ show that inhibition by lidocaine, tetracaine, dibucaine, and chlorpromazine is noncompetitive; slope and intercept replots are hyperbolic, showing that the inhibition is partial rather than complete.

Key Words: ATPase; F_1; local anesthetics; enzyme inhibition; benzocaine; lidocaine; tetracaine; dibucaine, chlorpromazine.

Introduction

The ability of the tertiary amine local anesthetics and tricyclic antipsychotic drugs to significantly inhibit mitochondrial ATPase at concentrations which are routinely attained under clinical conditions has, until recently, been largely unrecognized. Penefsky *et al.* (1960) showed that 50 μM chlorpromazine caused more than 50% inhibition of F_1-ATPase activity, but it appears that no further work was carried out in this area until the investigations of Palatini (1979, 1982) on antipsychotics, and the work from this laboratory on local anesthetics (Vanderkooi *et al.*, 1981; Chazotte *et al.*, 1982). Present evidence indicates that the effects of both of these classes of drugs on mitochondrial ATPase are qualitatively similar, with their inhibitory potencies differing approximately in proportion to their octanol/water partition coefficients (Chazotte *et al.*, 1982).

The possible implications of these observations for cellular metabolism

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have not been investigated in any detail as yet, but it is of interest to note that the inhibition of capping of lymphoctyes by local anesthetics is accompanied by a parallel decrease in the cellular ATP level (Montecucco *et al.,* 1981). Comparison of their findings with our results (Chazotte *et al.*, 1982) shows that the concentrations of anesthetics required to prevent capping are in the same range as will give an appreciable inhibition of ATPase activity. (The tacit assumption made here, that ATP synthetase activity is affected in a similar manner to the ATPase activity, must still be experimentally verified.) Since the mitochondrial electron transport chain is inhibited at several points by local anesthetics (Vanderkooi *et al.,* 1978; Chazotte and Vanderkooi, 1981), this effect may also contribute to the observed decrease in the cellular ATP level.

In our earlier work (Chazotte *et al.,* 1982) we showed that the ATPase activity of sonic submitochondrial particles (SMP) prepared from beef heart mitochondria and ATPase extracted from these particles by the method of Beechey *et al.* (1975) were both inhibited in a similar manner by several local anesthetics (*viz.,* procaine, lidocaine, tetracaine, and dibucaine), as well as by chlorpromazine (an antipsychotic and tranquilizer), propranolol (a β-adrenergic blocker and antiarrythmic), and procainamide (an antiarrythmic). All of these compounds contain a tertiary amine group and an aromatic ring system. For each of them except procainamide, the ATPase activity decreased hyperbolically as a function of inhibitor concentration; procainamide gave enzyme activation at low concentrations but inhibition at higher concentrations.

Two types of evidence were presented in support of the hypothesis that local anesthetics cause alterations in the tertiary or quaternary structure of chloroform ATPase (Chazotte *et al.,* 1982). First, the Schlieren pattern of ATPase in the analytical ultracentrifuge changed in shape from a smooth Gaussian profile to a sharpened form upon the addition of 1.0 mM tetracaine, and the sedimentation coefficient decreased slightly, from 13.95S to 13.05S. Second, the electrophoresis pattern on polyacrylamide gels changed from showing a single sharp band to a pattern with a shifted sharp band and one or more diffuse bands after preincubation with 1.0 or 2.0 mM tetracaine. These observations suggest that the anesthetics cause structural alterations on the quaternary level, but it has not yet been confirmed whether actual subunit dissociation occurs in free solution in the absence of the additional separating forces present in the gels. The rapid reversibility of inhibition by dilution (Chazotte *et al.,* 1982) is reason to suspect that subunit dissociation may not occur, as pointed out by Laikind *et al.* 1982).

An unusual feature noted in the anesthetic inhibition pattern was that the enzyme activity appeared to approach a nonzero plateau as the anesthetic concentration was raised. This was most evident in the case of tetracaine, for

which approximately 40% of the activity remains at tetracaine concentrations above 1.5 mM. The plateau for dibucaine is much lower, being only 5 to 10% of the original activity. Additional evidence is presented in this paper which shows that local anesthetics are partial noncompetitive inhibitors of chloroform ATPase.

Materials and Methods

Frozen beef heart mitochondria were obtained from The Institute for Enzyme Research, Madison, Wisconsin. Submitochondrial particles were prepared by sonication according to the method of Gregg *et al.* (1967); the SMP were stored at −20°C until used for ATPase preparation. The chloroform extraction method of Beechey *et al.* (1975) was used to make ATPase. The enzyme thus prepared had a maximal activity of about 14 μmol/min/mg protein, which is similar to values previously reported for this type of preparation (Linnett *et al.,* 1979). The ATPase was shown to be free of phospholipids by extracting an aliquot with a chloroform–methanol mixture (2:1, v/v), followed by thin-layer chromatography on silica gel plates. No phospholipid could be found; control experiments showed that as little as one or two moles of phospholipid per mole of protein would have been detectable.

Assay of ATPase activity was carried out using an ATP regenerating system similar to that described by Pullman *et al.* (1960). Our standard assay system (Chazotte *et al.,* 1982) consists of 50 mM Tris-acetate (pH 7.5), 4 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 0.5 mM ATP, 0.35 mM NADH, 8 units of lactate dehydrogenase, and 6.5 units of pyruvate kinase. An aliquot of F_1 and inhibitors are added as desired, to a final volume of 1.0 ml. All spectrophotometric assays were carried out in a Hitachi Model 110A spectrophotometer at 25°C. The rate of oxidation of NADH was followed at 350 or 355 nm since many of the anesthetics absorb at 340 nm. The method of Lowry *et al.* (1951) was used for protein determinations, with bovine serum albumin as the standard.

Results

pH Dependence of Enzyme Activity as a Function of Mg^{++} Concentration

The activity of ATPase is shown in Fig. 1A as a function of pH for three Mg^{++} concentrations, with the ATP concentration held at 0.5 mM. Maximal activity is obtained with the highest (4 mM) Mg^{++} concentration employed, and relatively low activity was found with equimolar Mg^{++} and ATP. The pH optimum was 8.0 for all three Mg^{++} concentrations. By contrast, Pullman *et*

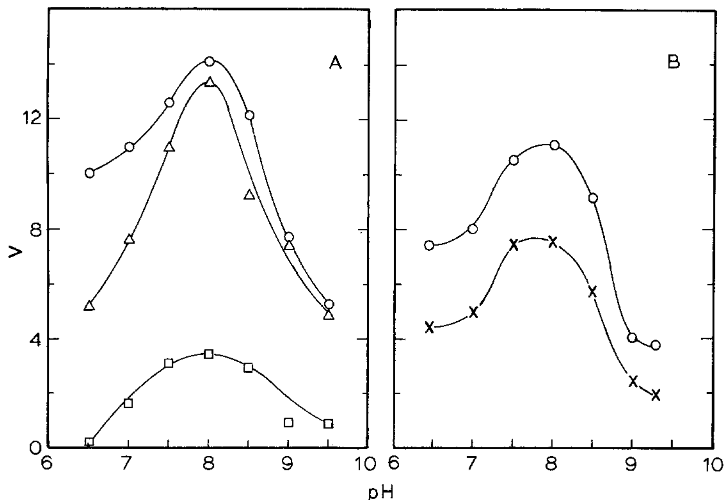

Fig. 1. pH dependence of F_1-ATPase activity. (A) Dependence on Mg^{++} concentration, with [ATP] held constant at 0.5 mM. (□) 0.5 mM Mg^{++}; (△) 1.25 mM Mg^{++}; (O) 4.0 mM Mg^{++}. Specific activity (V) is expressed as μmol ATP hydrolyzed/min/mg protein. (B) Dependence of activity on pH in the presence of benzocaine. (O) specific activity with 4.0 mM Mg^{++} and 0.5 mM ATP. (x) activity after adding 2.5 mM benzocaine. The experiments described in (A) and (B) were carried out on different days with different batches of F_1, which accounts for their difference in maximal activity.

al. (1960) reported a pH optimum for F_1-ATPase of 8.5–9.3, and maximal enzyme activity with an ATP-to-Mg^{++} ratio of 1.5. Also, Ulrich (1964) found that the pH optimum of rat liver ATPase shifted to a lower value as the Mg^{++} concentration was increased, but the optimum is insensitive to Mg^{++} in the preparation used here.

pH Dependence of Anesthetic Inhibition

A major difficulty encountered in studying the pH effects of local anesthetics is that the tertiary amine group present in most of these compounds has a pK_a between 8 and 9, and the solubility of the neutral alkaline form is usually too low to permit the measurement of potency over a wide pH range. In addition, even when data are obtained as a function of pH over the range in which the tertiary amine titrates, one cannot easily distinguish between pH effects due to the anesthetic and those resulting from titration of the protein itself. This problem is circumvented by using the uncharged local anesthetic benzocaine (p-aminobenzoic acid ethyl ester). Benzocaine is similar in structure to procaine but lacks the diethylamino group of the latter compound. Its aromatic amine has a low pK_a and hence is uncharged over the neutral and alkaline pH range.

It was shown by Chazotte (1981) that benzocaine inhibits succinate oxidase activity in submitochondrial particles in a similar manner to which this activity is inhibited by other local anesthetics (Chazotte and Vanderkooi, 1981). We have found that ATPase is also inhibited by benzocaine. Benzocaine is more potent than procaine as an ATPase inhibitor on a molar basis; 2.5 mM benzocaine gave 30% inhibition but 7.5 mM procaine was required for the same degree of inhibition. (Use of higher concentrations of benzocaine was precluded because of its limited solubility in water.) These findings suggest that the effects of local anesthetics on electron transport and on ATPase may be mechanistically more similar to nerve block than are the diverse calcium- and calmodulin-related effects of anesthetics, since the latter effects evidently require the presence of a positive charge (Singh *et al.*, 1983), whereas the uncharged benzocaine is an effective nerve blocking agent (Ritchie and Ritchie, 1968).

The pH dependence of ATPase inhibition by 2.5 mM benzocaine is shown in Fig. 1B. It may be noted here that inhibition occurs over the entire pH activity profile, but the relative degree of inhibition is somewhat greater at high and low pH than it is at the pH optimum (i.e., 40% inhibition at pH 6.5, 30% at pH 7.5, and 50% at pH 9.3). Since this pH dependence must be due to the titration of groups on the protein, it may be concluded that the enzyme is least sensitive to perturbation by this inhibitor near the pH of maximal activity.

ATPase Inhibition by Anesthetics Is Partial and Noncompetitive

Our earlier observation (Chazotte *et al.*, 1982) that local anesthetics appear to act as partial inhibitors of ATPase has been confirmed by preparing reciprocal plots of rate as a function of ATP concentration, at several anesthetic concentrations, followed by the replotting of the slope and intercept values of these graphs as a function of inhibitor concentration (Cleland, 1970).

Figures 2 through 5 show reciprocal plots for lidocaine, tetracaine, dibucaine, and chlorpromazine, respectively. The slopes from these graphs and their $1/V$ intercepts are replotted in Figs. 6 and 7 as a function of anesthetic concentration. The lines on the reciprocal plots appear to be parallel at low inhibitor concentration, which is usually taken to mean uncompetitive inhibition, but since an increase in slope is seen in all cases at higher concentrations, the inhibition type must be labeled as noncompetitive according to the definitions of Cleland (1970). All of the slope and intercept replots in Figs. 6 and 7 show curvature; most of these curves (i.e., all except the slope replots for dibucaine) are convex hyperbolic, which is the expected behavior for partial noncompetitive inhibition. The mechanistic description offered by Cleland (1970) for partial inhibition is that "the inhibitor causes

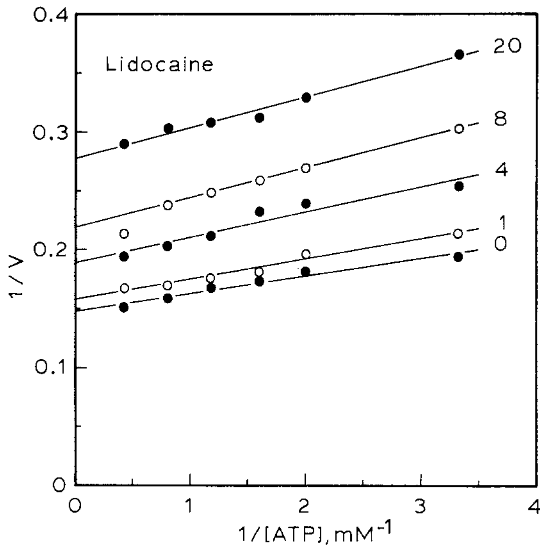

Fig. 2. Plot of $1/V$ vs. $1/[ATP]$ for inhibition by lidocaine. The concentrations of inhibitors are given on the least-squares lines in millimolar units. Assay conditions (except for variable [ATP]) were as given in the Materials and Methods section.

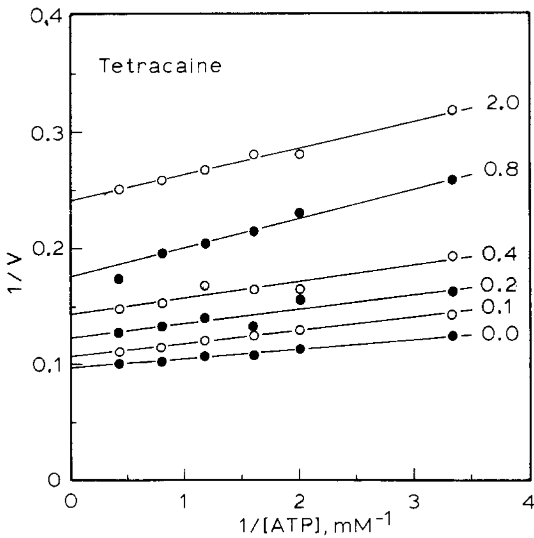

Fig. 3. Plot of $1/V$ vs. $1/[ATP]$ for inhibition by tetracaine. Notation as in Fig. 2.

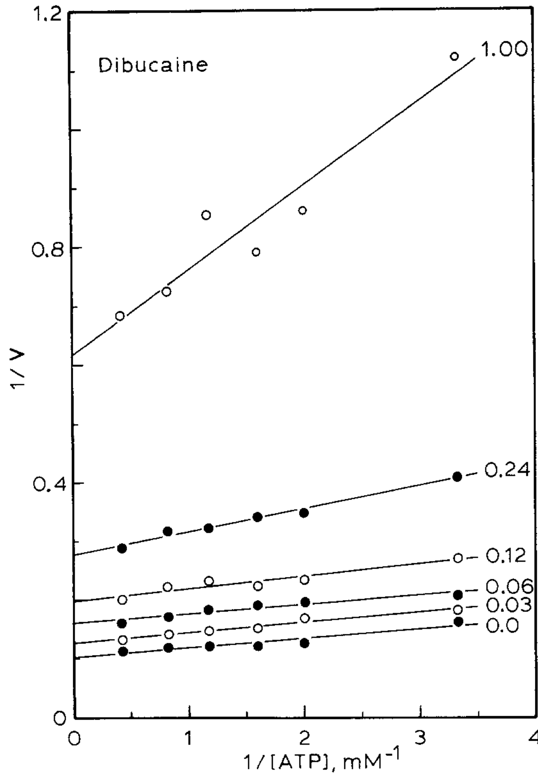

Fig. 4. Plot of $1/V$ vs. 1/[ATP] for inhibition by dibucaine. Notation as in Fig. 2.

the reaction flux to be diverted to an alternate pathway that is slower than the normal one."

Discussion

The effects of anesthetics on chloroform ATPase somewhat resemble those of aurovertin, which is a partial inhibitor of F_1-ATPase (Ebel and Lardy, 1975). With MgATP as the substrate, aurovertin gave uncompetitive inhibition as indicated by parallel lines on its reciprocal plot, but the replot of the intercepts was convex hyperbolic, which confirmed that the inhibition was partial rather than total. Fluorescence studies on aurovertin binding to F_1 showed that one or two moles aurovertin were bound per mole of F_1, depending upon the conditions (Chang and Penefsky, 1973).

Other inhibitors have also been found to give noncompetitive or uncom-

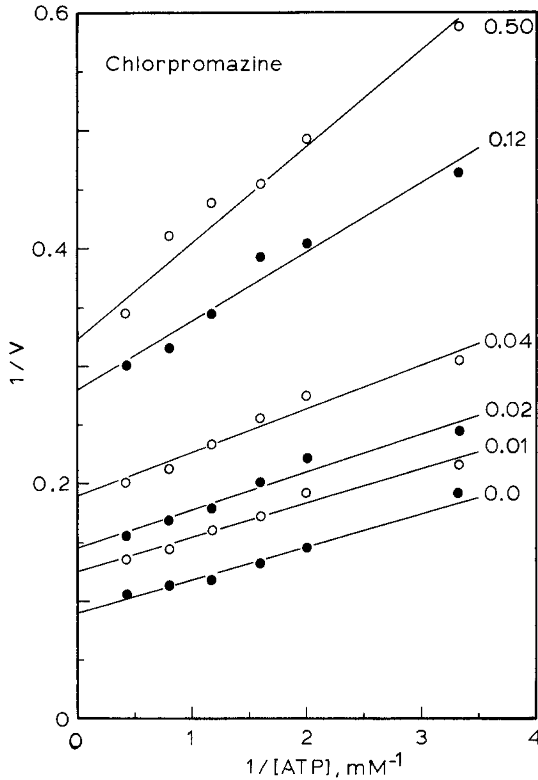

Fig. 5. Plot of $1/V$ vs. $1/[ATP]$ for inhibition by chlorpromazine. Notation as in Fig. 2.

petitive inhibition. Lenaz *et al.* (1978) reported that the secondary amine general anesthetic ketamine gave uncompetitive inhibition, and Palatini (1982) showed that fluphenazine, a tricyclic antipsychotic, gave noncompetitive inhibition. Insufficient data were presented in these cases to determine whether the inhibition was partial or total. Laikind *et al.* (1982), on the other hand, reported that chlorpromazine caused competitive inhibition of F_1-ATPase at low concentration, but gave uncompetitive inhibition at higher concentrations. They proposed a competitive catalytic site mechanism for anesthetic inhibition on the basis of these data. They have subsequently modified this proposal, however, as a result of their studies with quinacrine, another tricyclic compound (Laikind and Allison, 1983). This compound appeared to give competitive inhibition when the substrate was MgATP, but uncompetitive inhibition when an excess constant concentration of Mg^{++} was used. Neither this observation nor the several reports of noncompetitive and uncompetitive inhibition by the other workers referred to above can be accounted for by a simple classical competitive inhibition model.

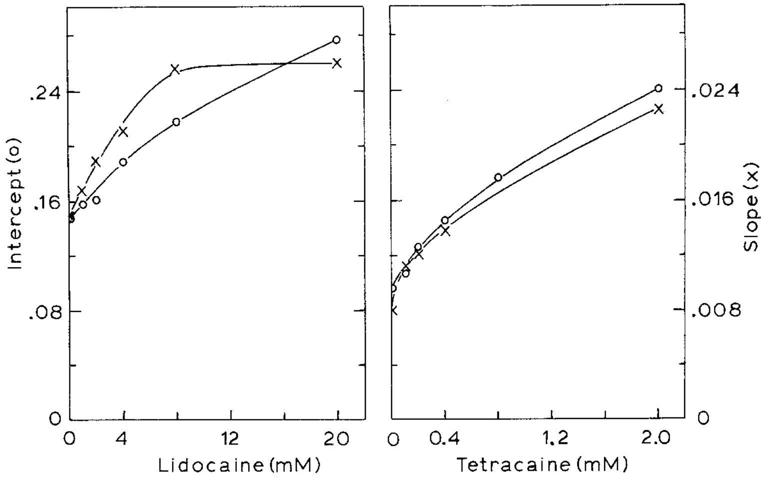

Fig. 6. Slope and intercept replots of the data given in Figs. 2 and 3 (O) intercepts on the $1/V$ axes, replotted as a function of inhibitor concentration (left axes). (x) slopes of the reciprocal plots (right axes).

The partial noncompetitive inhibition given by anesthetics and other compounds may be the result of specific site binding which causes the reaction coordinate to shift to a slower route; alternatively, the inhibitors may be interacting at multiple weak binding sites, thereby causing a protein conformational perturbation which diminishes but does not abolish the efficacy of the enzyme. It is likely that aurovertin acts by the first of these two mechanisms, considering the evidence that only one or two molecules bind to

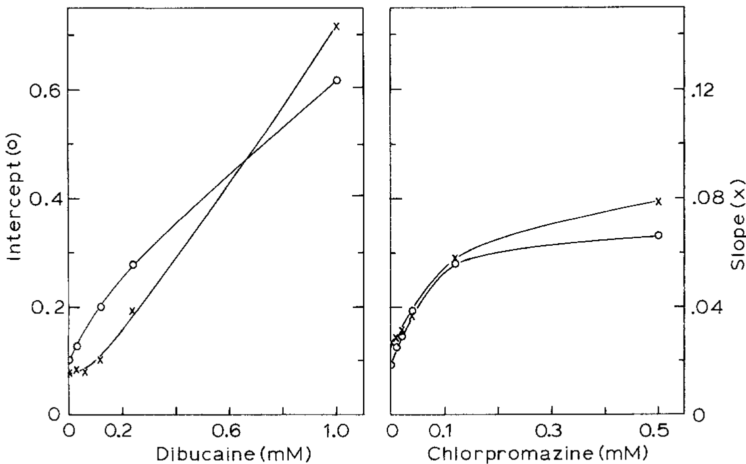

Fig. 7. Slope and intercept replots of the data given in Figs. 4 and 5. Notation as in Fig. 6.

F_1 (Chang and Penefsky, 1973). No data are yet in hand on the stoichiometry of local anesthetic or tricyclic antipsychotic noncovalent binding to ATPase that might show whether there are few or many sites at which they interact, or how strongly. The possibility cannot be overlooked that these compounds may act as general nonspecific chaotropic agents, considering that alcohols also cause ATPase inhibition (Vanderkooi *et al.,* 1981). There are significant differences between the characteristics of alcohol and anesthetic inhibition, however; butanol gives complete rather than partial inhibition, and butanol and benzyl alcohol inhibition have both been found to have a time-dependent, irreversible component (Vanderkooi *et al.,* 1981, and unpublished studies). Local anesthetic inhibition, on the other hand, appears to be time-independent and reversible.

Another conceivable mechanism which should be mentioned is that different molecular forms of ATPase may be present and may have different sensitivities to inhibition by anesthetics. Fisher *et al.* (1981) have shown that ATPase prepared by the chloroform method actually contains two separable enzymes, one of which has an additional subunit not present in the F_1 originally described by Pullman *et al.* (1960). The two forms differ in specific activity and other properties. Both of these are presumably present in our preparation. The chance seems small that only one of these forms is being inhibited by local anesthetics, however. If anesthetics acted as complete inhibitors of one of the forms but did not affect the other form, one should expect that all anesthetics would give the same maximal degree of partial inhibition, but this is not the case. With tetracaine there is a residual enzyme activity of the order of 40% of the original, but much less than this remains with chlorpromazine and dibucaine. Hence we are left with the alternative explanations mentioned earlier, that anesthetics and related molecules cause partial inhibition by changing the reaction pathway or by changing the tertiary or quaternary structure of the enzyme; these options are not mutually exclusive.

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