Biochimica et Biophysica Acta, 1015 (1990) 248–252
Elsevier

BBABIO 43140

Mechanism of local anesthetic effect. Involvement of F_0 in the inhibition of mitochondrial ATP synthase by phenothiazines

F. Dabbeni-Sala and P. Palatini

Dipartimento di Farmacologia, Università di Padova, Padova (Italy)

(Received 22 May 1989)

Key words: Mitochondrial ATP synthase; ATP synthase; Phenothiazine; Anesthetic mechanism

The mechanism whereby tertiary amine local anesthetics affect the activity of membrane proteins was investigated by studying the interaction of phenothiazines with mitochondrial ATP synthase. These drugs caused inhibition of the activity of the membrane-bound enzyme at concentrations that do not perturb the phospholipid bilayer. The inhibitory effect appeared consequent to interaction with multiple sites located on both the F_1 and the F_0 components of the enzyme complex, since: (a) Dixon plots were parabolic; (b) the membrane-bound enzyme was more sensitive to the drug effect than the isolated F_1 component; (c) conditions that decreased oligomycin sensitivity also decreased the sensitivity to phenothiazines; (d) irreversible binding of photochemically activated phenothiazines to the ATP synthase complex, followed by detachment of the F_1 moiety and reconstitution with purified F_1 resulted in an inhibited enzyme complex. These data are interpreted as indicating that tertiary amine local anesthetics affect the activity of membrane proteins by interacting with hydrophobic sites located on both their integral and peripheral domains.

Introduction

The mode of interaction of tertiary amine local anesthetics with membrane proteins remains a controversial matter. Direct drug–protein interactions with either the peripheral moiety [1,2] or the lipid embedded sector [3–5], or indirect effects through a perturbation of the phospholipid bilayer [6,7] have been proposed as possible mechanisms. The mitochondrial ATP synthase represents an ideal model system for discriminating among these possibilities, since it offers the opportunity of comparing a drug effect on the whole, membrane-bound, complex with that on the isolated, hydrophylic, F_1 moiety. The observation that phenothiazines and other tertiary amine local anesthetics affect in a qualitatively identical manner the activities of the soluble lipid-free enzyme and the membrane-bound complex has been taken as evidence of a selective drug interaction with the F_1 component [2,8]. Subsequent studies have therefore explored in detail the mode of interaction of local anesthetics with F_1 [9–12]. However, it has also been reported that the membrane-bound enzyme exhibits a greater sensitivity to the inhibitory effect of various local anesthetics than the isolated F_1 moiety [2,8,13]. This is suggestive of an effective drug interaction also with the membranous moiety of the enzyme. We therefore decided to reinvestigate the mechanism of the local anesthetic effect on mitochondrial ATP synthase by examining the interaction of two phenothiazines, trifluoperazine (TFP) and chlorpromazine (CPZ) with two differently purified ATP synthase preparations, that of Serrano et al. [14] and Complex V of Stiggal et al. [15]. Phenothiazines were chosen as anesthetic agents since, upon photoactivation in the presence of the enzyme, they convert to free radicals that react irreversibly with the parent drug binding site(s) and are therefore useful in identifying the affected moiety [16,17]. Complex V and the preparation of Serrano et al. [14] were chosen since they are two highly purified and well-characterized ATP synthase preparations. They contain the F_0-F_1 complex in a virtually lipid-free, inactive, form that can be reactivated by insertion into phospholipid liposomes. They differ for the presence in the Serrano et al. [14] preparation of the adenine nucleotide carrier, which, according to some authors, is functionally related to the ATP synthase complex (see Ref. 18 for a review) and, as a matter of fact, has a striking influence on its mode of interaction

Abbreviations: TFP, trifluoroperazine; CPZ, chlorpromazine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SMP, submitochondrial particles.

Correspondence: F. Dabbeni-Sala, Dipartimento di Farmacologia, Università di Padova, Largo Meneghetti 2, 35131 Padova, Italy.

with phospholipids [19–21]. These preparations present over submitochondrial particles the advantage of a known protein composition and offer the opportunity of evaluating a possible influence of the composition of the phospholipid bilayer on the drug effect.

Materials and Methods

Materials

ATP, phospho*enol*pyruvate, chlorpromazine · HCl, trifluoperazine · 2HCl and all buffers were obtained from Sigma. Phosphatidylcholine was obtained from egg yolk as previously described [21]. HPLC-purified phosphatidylserine from bovine brain was a kind gift from Fidia (Abano Terme, Italy).

Reconstitution procedures

ATP synthase complexes were prepared from bovine heart MgATP submitochondrial particles as originally described [14,15] and reconstituted with phospholipids according to the cholate-dialysis method [22]. In particular, the delipidated complexes were reconstituted with 3 μmol/mg protein of sonicated liposomes of either egg phosphatidylcholine or egg phosphatidylcholine plus 10% phosphatidylserine. Cholate was removed by overnight dialysis against 1000 ml of 25 mM Tris-Mes (pH 7.4) at 0 °C. F_0 was obtained from the Serrano et al. [14] preparation by treatment with 3.5 M NaBr exactly as described by Tzagoloff et al. [23]. NaBr was used to this purpose since, unlike urea, it does not cause detachment of OSCP [23]. Reassembly of F_0-F_1 complex was carried out with F_0 previously reconstituted with negatively charged phospholipids and F_1 prepared according to Horstman and Racker [24]; F_0 and F_1, in a protein ratio of 5 to 1, were incubated for 10 min at 37 °C in 25 mM Tris-Mes (pH 7.4). This F_0/F_1 ratio ensures complete rebinding of F_1 to F_0 [22].

Phenothiazine photoactivation

100–200 μg of enzyme preparation were incubated 10 min in 100–200 μl 25 mM Tris-Mes under red safety light at room temperature in the presence of TFP or CPZ. The samples were then irradiated at 256 nm for 15 min with a UV SL56 Mineral light lamp (S. Gabriel, CA). Proteins were recovered by centrifugation at 180000 × *g* for 30 min at 0 °C and the pellet was resuspended in 100 μl Tris-Mes (pH 7.4).

Analytical procedures

ATPase activity was measured as the release of ADP by monitoring spectrophotometrically at 340 nm the NADH oxidation in a coupled lactate dehydrogenase-pyruvate kynase ATP regenerating system, at 37 °C [15]. When oligomycin-treated samples were assayed, 2 μg oligomycin per ml was added to the couvette. Preliminary experiments had demonstrated that TFP and CPZ, either before or after photoactivation, did not interfere with the effect of oligomycin, since they modified neither the IC_{50} nor the time-course of oligomycin inhibition.

The SDS-PAGE of Serrano et al. [14] ATP synthase and F_0 preparations was performed according to the method of Montecucco et al. [20]. Briefly, the enzyme pellets were dissolved in 4% SDS, 3% β-mercaptoethanol, 10 mM Tris-acetate (pH 8.2) and subjected to electrophoresis on 20 cm long polyacrylamide linear gradient (13–19%) gels containing a linear sucrose gradient (0–24%). After staining with Coomassie Blue, the gel patterns were recorded by scanning with a Shimadzu Dual Chromato Scanner CS-930 at 570 nm. Protein concentration was determined according to Lowry et al. [25].

IC_{50} values reported (inhibitor concentrations giving 50% inhibition) are the means of four separated determinations. The standard error of the mean never exceeded 5%.

Results and Discussion

The kinetics of inhibition of the ATPase activity by TFP is shown in Fig. 1 in the form of Dixon plots. Qualitatively identical inhibition patterns were observed with CPZ. The upward curvature of the plots indicates that the inhibition was consequent to interaction of the drug with multiple inhibitor sites [26]. From Fig. 1 it is also apparent that the nature of the inhibition was influenced neither by the method of purification of the enzyme nor by the charge of the phospholipid bilayer. IC_{50} values for TFP and CPZ are reported in Table I. They are significantly lower than those previously observed with F_1 (e.g., 70 μM for CPZ, [2,13]) and similar to those obtained with SPM [2,8], confirming the greater sensitivity of the whole, membrane bound, enzyme than the isolated F_1. In principle, this may be due to three

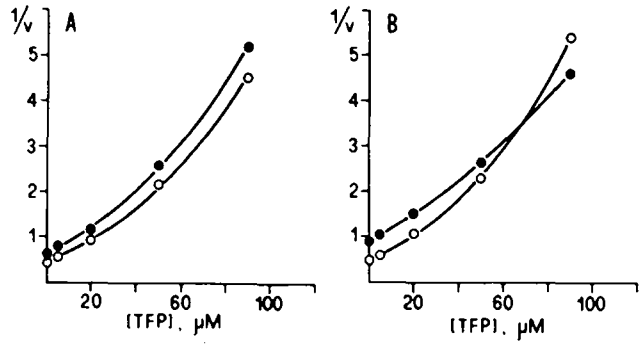

Fig. 1. Dixon plots of TFP inhibition of ATPase activity. Enzyme velocity is expressed as μmol/min per mg protein. (A) Serrano et al. [14] preparation. (B) Complex V. ●, Enzyme complex reconstituted with egg phosphatidylcholine (egg-PC). ○, Enzyme complex reconstituted with egg phosphatidylcholine plus 10% phosphatidylserine (egg-PC + 10% PS).

possibilities: (1) indirect effects through an alteration of the phospholipid bilayer; (2) drug interaction also with F_0; (3) a different, more drug-sensitive, conformation of F_1 when bound to its membrane sector. The first possibility seems to be excluded, since perturbations of the phospholipid bilayer have been observed only at phenothiazine concentrations (50 or 500 μM [3,27]) greater that those found effective in this and previous studies [2,8]. The second possibility is suggested by the observation (see Table I) that complex V exhibited a lower sensitivity to the inhibitors when reconstituted with isoelectric phospholipids. As previously observed [21], in these conditions Complex V exhibited also a reduced sensitivity (51%) to oligomycin, a drug that causes inhibition of the catalytic activity of F_1 by interacting exclusively with F_0. The preparation of Serrano et al. [14], that retained virtually full oligomycin sensitivity (95%) upon reconstitution with isoelectric phospholipids, maintained unaltered sensitivity to the phenothiazines. It is thus conceivable that, as proposed for oligomycin and DCCD [28], these drugs induce a conformational change in F_0 that is transmitted to F_1. This conformational change appears, however, consequent to interaction with site(s) distinct from that of oligomycin, since no interference has been observed between the inhibitory effects of oligomycin and phenothiazines (see Methods). Although a higher phospholipid-to-protein ratio (20 μmol/mg protein) ensures a better coupling between the F_0 and F_1 components of Complex V [21], such a phospholipid concentration could not be used since it prevented the inhibitory effects of phenothiazines (experiments not shown). This prevention of the inhibition, that is still more pronounced with negatively charged liposomes, is due to the sequestration of the highly hydrophobic drug molecules by phospholipids [29,30]. To discriminate unambiguously between the second and third possibilities, we studied the effect of phenothiazines also upon photoactivation of the drugs in the presence of the enzyme. This procedure results in the formation of free radicals that react irreversibly with the parent drug binding sites. If inhibitory sites are present also on F_0, then detachment of F_1 from the free-radical-treated complex and reconstitution of the F_1-depleted enzyme with purified, untreated, F_1 should result in an enzyme complex exhibiting reduced activity with respect to a control preparation subjected to the same procedure in the absence of the drugs. The preparation of Serrano et al. [14] was used for these experi-

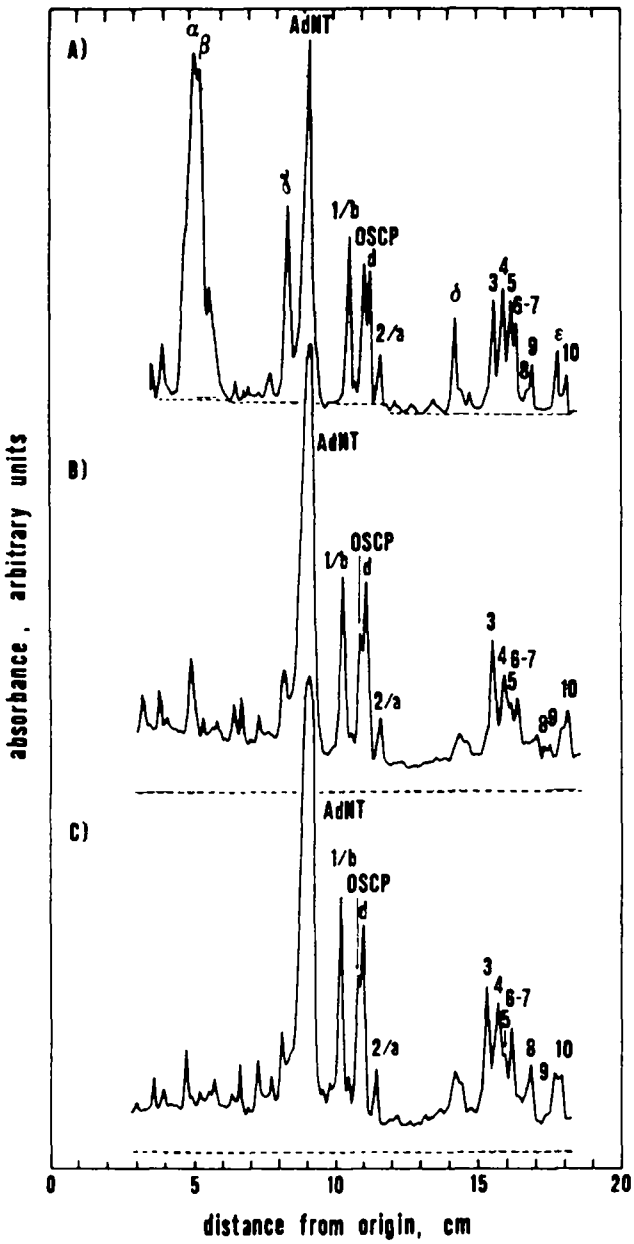

Fig. 2. SDS-PAGE patterns of (A) Serrano et al. [14] ATP synthase; (B) F_0 obtained from the Serrano et al. [14] ATP synthase by treatment wth NaBr; (C) F_0 obtained by NaBr treatment of Serrano et al. [14] ATP synthase previously irradiated in the presence of 12 μM TFP. Experimental procedures are described under Materials and Methods. Greek letters indicate the subunits of F_1. Numbers denote the bands found associated with F_0, according to the nomenclature of Montecucco et al. [20]. In the 18–25 kDa region, the nomenclature of F_0 subunits according to Walker et al. [33] is also given. AdNT and OSCP denote Adenine nucleotide carrier and oligomycin sensitivity conferring protein, respectively. Subunits 4, 5, 6, and 8 are also referred to as ATPase inhibitor, F_6, A6L, and c (DCCD binding protein), respectively.

TABLE I

IC_{50} values for the inhibition of phospholipid-reconstituted mitochondrial ATP synthase complexes by TFP and CPZ

For abbreviations see legend to Fig. 1.

ATP synthase complex	IC_{50} (μM)	
	TFP	CPZ
Egg PC Serrano ATP synthase	19.7	39.2
Egg PC + 10% PS Serrano ATP synthase	17.2	30.8
Egg PC Complex V	30.5	56.0
Egg PC + 10% PS Complex V	18.0	36.0

ments, since complete detachment of F_1 and full preservation of OSCP can not be obtained with Complex V [31]. It was preliminary verified that phenothiazine free radicals actually interact with the same binding sites as their parent drugs. This was demonstrated by the two following observations (Table II). (a) IC_{50} values for the free radicals of CPZ and TFP were in the same ratio as those of their parent drugs (cf. Table I), i.e., TFP was more effective than CPZ. If the inhibition were not due to photoactivation of drug molecules occupying the inhibitor sites but to interaction with aspecific sites of radicals generated in free solution, then CPZ, the free radical of which has a longer half-life [32], should prove a more potent inhibitor than TFP. (b) As previously observed for the reversible inhibitory effect of unmodified phenothiazines [8,13], also the irreversible inhibitory effect of photoactivated phenothiazines increased upon exposure of the enzyme to Mg^{2+} in the presence of ATP. Table III shows that the activity of an enzyme preparation irradiated in the presence of 12 μM TFP is 85% inhibited with respect to that of the same enzyme preparation irradiated in the absence of the drug. When the free-radical-treated and untreated preparations were depleted of their F_1 moieties and reconstituted with purified F_1, the activity of the reconstituted complex previously exposed to the action of TFP free radicals was still 48% inhibited. As shown in Fig.2, SDS-PAGE patterns of free-radical-treated and untreated F_0 preparations were overlapping and revealed complete detachment of F_1 and loss of none of the F_0 subunits. These observations and the full sensitivity to oligomycin of the measured ATPase activity (Table III) indicate complete rebinding of added F_1 to a fully preserved F_0 moiety and demonstrate that the residual inhibition of the reconstituted enzyme previously irradiated in the presence of TFP is due only to drug interaction with F_0.

TABLE II

IC_{50} values for the irreversible inhibition of ATPase activity by photoactivated phenothiazines

The preparation of Serrano et al. [14], reconstituted with negatively charged phospholipids, was exposed to UV light in the absence or presence of different concentrations of TFP or CPZ, centrifuged and resuspended as described in detail under Materials and Methods. In some experiments, before exposition to photoactivated drugs, the phospholipid-reconstituted enzyme complex was incubated for 5 min at 37°C in a medium containing 25 mM Tris-Mes (pH 7.4), 2 mM $MgCl_2$, 1 mM ATP and an ATP-regenerating system composed of 4 mM phospho*enol*pyruvate plus 20 μg pyruvate kinase. Proteins were then recovered by centrifugation at 180000 × *g* for 30 min and resuspended in 25 mM Tris-Mes (pH 7.4).

Prior treatment	IC_{50} (μM)	
	TFP	CPZ
None	5.5	12.0
Preincubation with 2 mM $MgCl_2$/1 mM ATP	3.0	6.5

TABLE III

Effect on ATPase activity of the irreversible binding of TFP to F_0

The preparation of Serrano et al. [14] was irradiated in either the absence or presence of 12 μM TFP as described under Methods. Aliquots of both the free-radical-treated and untreated enzymes were reconstituted with negatively charged phospholipids and their activities were determined. The remaining aliquots were depleted of F_1 by treatment with NaBr (see Materials and Methods), reconstituted with negatively charged phospholipids and tested for ATPase activity either before or after reassembly of the F_0-F_1 complex with purified F_1 also as described under Materials and Methods.

Enzyme preparation	ATPase activity (μmol/mg per min)	% inhibition by TFP free radical	Oligomycin sensitivity (%)
Serrano et al. ATP synthase	9.90	-	95
Free-radical-treated Serrano et al. ATP synthase	1.50	85	95
NaBr-F_0	0.07	-	-
Free-radical-treated NaBr-F_0	0.07	-	-
NaBr-F_0 + F_1	5.60	-	94
Free-radical-treated NaBr-F_0 + F_1	2.60	48	90

In conclusion, this study has shown that phenothiazines affect the ATPase activity of mitochondrial ATP synthase by interacting with multiple inhibitor sites located on both the F_1 and F_0 components of the enzyme. This is inferred from the following observations: (a) Dixon plots are upwardly curved; (b) the drugs are more effective inhibitors of the membrane-bound ATP synthase complex than of the isolated F_1; (c) conditions that decrease the sensitivity to oligomycin reduce the sensitivity to the phenothiazines as well; (d) irreversible binding of phenothiazines to the enzyme complex followed by detachment of F_1 and reconstitution with purified F_1 results in an inhibited enzyme complex. The foregoing results are of potential significance to the general question of the site of anesthetic action. The observations that phenothiazines interact with both the extrinsic and the membrane-bound moieties of mitochondrial ATP synthase and both types of interaction contribute to the drug effect suggest that the activity of local anesthetic agents on membrane proteins is not mediated by a selective interaction with either of these moieties. Both the peripheral and the integral components appear instead to be pharmacologically relevant targets.

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