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PHENOTHIAZINES AND RELATED COMPOUNDS DISRUPT MITOCHONDRIAL ENERGY PRODUCTION BY A CALMODULIN-INDEPENDENT REACTION

LARRY RUBEN and HOWARD RASMUSSEN

Department of Internal Medicine, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510 (U.S.A.)

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Phenothiazines and related compounds bind to mitochondrial membranes in approximate proportion to their affinities for calmodulin. Penfluridol (16 μM), pimozide (20 μM), or trifluoperazine (66 μM) completely inhibit ADP-stimulated respiration in isolated rat liver mitochondria, but exert no effect on either uncoupler- or Ca^{2+}-stimulated respiration. The inhibition of ADP-stimulated respiration results from inhibition of the oligomycin-sensitive ATPase. Inhibition of the ATPase does not involve interaction of phenothiazine with calmodulin. The addition of calmodulin with or without calcium to mitochondrial inner membrane preparations has no effect on ATPase activity. The addition of EGTA and the ionophore A23187 prior to the addition of phenothiazine does not prevent the phenothiazine-induced inhibition of the ATPase. Measurements of inner membrane calmodulin content by gel electrophoresis or cyclic nucleotide phosphodiesterase activation are negative. Despite the absence of calmodulin in the inner membrane preparations, 12.5 nmol trifluoperazine bind per 100 μg of membrane protein with an association constant, K, of $6.5 \cdot 10^4$ M^{-1}. We conclude that calmodulin-binding neuroleptic agents, when added to whole cells, have the potential to disrupt mitochondrial energy production by a reaction which apparently does not involve a phenothiazine-calmodulin interaction.

Calmodulin is a Ca^{2+} receptor protein that is distributed ubiquitously among eucaryotic cells. Using cell homogenates and purified proteins, calmodulin has been shown to regulate a large number of well defined enzymatic reactions [1–3]. In order to evaluate fully the role of calmodulin in the regulation of specific cell functions, studies on whole cells are necessary. Such studies are hampered by the lack of selective probes to inhibit calmodulin activity. A great deal of enthusiasm has been generated, therefore, by the discovery that phenothiazines and related compounds are high-affinity ligands for calmodulin [4,5] which inhibit its regulatory functions. Based on the premise that the effects produced by phenothiazines on isolated cells result from a calmodulin-phenothiazine interaction, calmodulin has been implicated in numerous cellular processes. These conclusions are valid only if phenothiazines bind specifically to calmodulin and exert no effects on other components of the cell. However, on the basis of known properties of phenothiazines and related compounds, binding to loci other than calmodulin is a distinct possibility.

Phenothiazines are hydrophobic molecules which bind to calmodulin in the presence of Ca^{2+}, when hydrophobic groups on the calmodulin are exposed [5,7]. The hydrophobic nature of phenothiazines suggests the potential for nonspecific interactions with hydrophobic cellular components. For example,

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

chlorpromazine binding of comparative high affinity to artificial and natural membranes has already been described [8,9]. Inhibition of mitochondrial respiration by dihydroxylated chlorpromazine derivates has also been reported [10]. In the present report, we describe high-affinity binding of penfluridol, pimozide and trifluoperazine to mitochondrial inner membrane. This binding leads to inhibition of a specific respiratory function. All three compounds inhibit the oligomycin-sensitive ATPase of the inner mitochondrial membrane with high affinity in a manner that apparently does not involve calmodulin. Nevertheless, the concentrations needed to produce this inhibitory effect are similar to those needed to block several calmodulin-regulated reactions.

Materials and Methods

Materials

Penfluridol and pimozide were obtained as gifts from M.F. Ralston of McNeil Laboratories. Trifluoperazine, [^{3}H]trifluoperazine and trifluoperazine sulfoxide were kindly provided by E. Gallagher of Smith, Kline and French Laboratories. [2,8-^{3}H]ADP was purchased from New England Nuclear. Precoated plastic-backed polyethylimine-cellulose F sheets (0.1 mm thick) were purchased from MC/B Manufacturing, Cincinnati, OH. All other biochemicals were reagent grade and purchased from major suppliers.

Methods

Mitochondria were isolated from the livers of male Sprague-Dawley rats (200–300 g). The mitochondria were prepared in H medium containing 0.5 mM EGTA using a modification of standard procedures [11], in which the nuclei were pelleted at 650 $\times g$ for 10 min and the mitochondria were pelleted at 8000 $\times g$ for 10 min. The mitochondrial pellet was washed once in H medium with EGTA and once in H medium without EGTA. Gradient mitochondria were prepared by adding 15 mg mitochondrial protein to 40-ml tubes containing 35–50% (w/w) sucrose gradients with 10 mM Tris-HCl, pH 7.4, throughout. The gradients were centrifuged at 50000 $\times g$ for 2 h. Lubrol-treated preparations of mitochondrial inner membrane were prepared as described by others [12]. Protein was determined by a modified Biuret procedure [13] or by the method of Lowry et al. [14].

Calmodulin was quantitated in liver fractions by resuspending the fraction at 10 mg/ml in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4. Following the addition of Ca^{2+} (100 μM), the mixture was heated in a boiling water bath for 1 min. The supernatant was passed through a small DEAE-cellulose column, washed once with 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, and washed again with 0.4 M NaCl, 0.05 M Tris-HCl, pH 7.4. The final wash was dialyzed overnight against distilled water and was lyophilized to dryness. The dried mitochondrial extract was resuspended in a small volume of 0.5 M Tris-HCl, pH 7.4, and was assayed for its ability to stimulate brain cyclic nucleotide phosphodiesterase by the one-step procedure described previously [15].

Polyacrylamide gel electrophoresis of the mitochondrial extract was performed in 7–15% discontinuous gels containing SDS using the buffers of Laemmli [16]. Samples were preincubated with 500 μM Ca or 5 mM EGTA prior to electrophoresis.

Mitochondrial respiratory activity was determined with a Clark-type oxygen electrode attached to a YSI oxygen monitor, thermostatically maintained at 27°C. Continuous recordings were made of the electrode response.

Mitochondrial adenine nucleotide pools were determined in mitochondria which were preincubated for 1 min in 3 ml of medium containing 5 mM succinate, 1 mM P_i, 1 mM Mg^{2+}, 210 mM mannitol, 70 mM sucrose, 0.05% bovine serum albumin, 2 mM Hepes (pH 7.0) and 0.5 mM ADP. The appropriate calmodulin-binding agent was added, followed 30 s later by 1.8 mM ADP (8.4 μCi/mmol; [2,8-^{3}H]ADP). After 1 min incubation, 0.8 μM oligomycin and 80 μM atractyloside were added to stop the reaction. An aliquot of the reaction mixture was filtered, washed, extracted in 6% $HClO_4$, and was neutralized with 3 M K_2CO_3. Labeled ATP and ADP were separated by polyethylinine-cellulose chromatography [17] while the ATP and ATP + ADP content of the extract were determined using the luciferin/luciferase assay as described previously [18].

ATP activity was determined in Lubrol-treated inner membranes by measuring the amount of phosphate generated [19] following 20 min incubation at 30°C. The incubation medium contained 10 μg inner membrane protein in 1 ml of 250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 1 mM $MgSO_4$, 5 U pyruvate

kinase, 2.5 mM phospho*enol*pyruvate and 3 mM ATP. The reaction was started by the addition of ATP and stopped by the addition of 5% trichloroacetic acid.

The binding of [^{3}H]trifluoperazine to Lubrol-treated mitoplasts (100 μg/ml) was quantitated after 90 min incubation at 25°C in 100 μM Ca^{2+}, 170 mM sucrose, 210 mM mannitol, 2 mM Hepes, pH 6.0. [^{3}H]Trifluoperazine (60 μCi/μmol) was added. Aliquots were filtered, washed and counted to determine binding. The addition of a 100-fold excess of unlabeled trifluoperazine completely blocked binding of [^{3}H]trifluoperazine to the membrane. This procedure was likely to underestimate the affinity and number of binding sites in the membranes for trifluoperazine, since the membranes were washed before counting.

Results

Interaction of phenothiazines and related compounds with mitochondria

The interaction of phenothiazines with mitochondria was initially studied using [^{3}H]trifluoperazine as a probe. Mitochondria were preincubated with 60 μM [^{3}H]trifluoperazine (65 μCi/μmol) and then separated on a sucrose gradient. As depicted in Fig. 1, [^{3}H]trifluoperazine migrated with the mitochondrial fraction, as determined by the distribution of protein, succinate dehydrogenase and radioactivity.

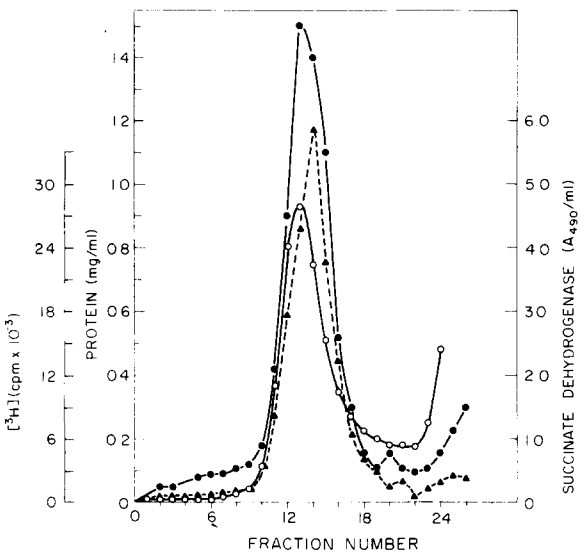

Fig. 1. Sucrose density gradient centrifugation of rat liver mitochondria. Mitochondria (15 mg) were preincubated in the presence of 60 μM [^{3}H]trifluoperazine (60 μCi/mol), layered over sucrose gradients (35–50%, w/w) and centrifuged at 50 000 X *g* for 2 h. Protein (●———●), CPM [^{3}H]-trifluoperazine (○———○) and succinate dehydrogenase (▲———▲) were assayed. Succinate dehydrogenase was quantitated as the absorbance at 490 nm of reduced *p*-iodonitrotetrazolium violet, extracted into ethyl acetate [22].

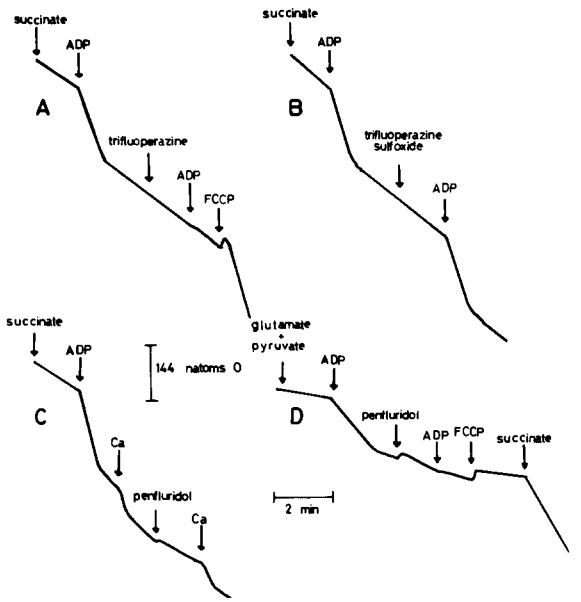

Fig. 2. Effects of calmodulin-binding agents on mitochondrial respiration. Mitochondria (0.8 mg/ml) were incubated at 27°C in 3 ml of medium containing 70 mM sucrose, 210 mM mannitol, 0.05% bovine serum albumin, 1 mM Mg^{2+}, 1 mM P_i, 2 mM Hepes (pH 7.0). Oxygen consumption was monitored with a Clark-type oxygen electrode connected via a YSI oxygen monitor to a recording unit. Succinate (5 mM), malate (5 mM), pyruvate (5 mM), ADP (0.1 mM), trifluoperazine (66 μM), penfluridol (16 μM), trifluoperazine sulfoxide (1 mM) or Ca^{2+} (50 μM) were added to the incubation medium as indicated.

In order to establish whether or not this binding influenced mitochondrial respiratory function, mitochondria were incubated under conditions where oxygen consumption could be monitored. As indicated in Fig. 2A, the addition of 66 μM trifluoperazine to respiring mitochondria completely inhibited further ADP-stimulated respiration when succinate served as the substrate. Penfluridol (16 μM) and pimozide (20 μM) produced similar effects, being different only in that they also inhibited state 4 respiration (data not shown). By contrast, uncoupler-stimulated respiration (FCCP or 2,4-dinitrophenol) was not

inhibited, indicating that none of the compounds tested affected electron transport directly. At higher concentrations, however, uncoupler-stimulated respiration was inhibited, suggesting the occurrence of gross membrane changes. The sulfoxide derivative of trifluoperazine is hydrophilic and does not interact substantially with calmodulin [4]. Its addition did not lead to an inhibition of respiration, even at concentrations as high as 1 mM (Fig. 2B). The interaction of phenothiazine with mitochondria, therefore, has the same specificity as the interaction with calmodulin.

The inhibition by calmodulin-binding agents was in many ways specific for ADP-stimulated respiration. As shown in Fig. 2C, Ca^{2+}-stimulated respiration was not inhibited by quantities of penfluridol sufficient to inhibit completely ADP-stimulated respiration.

The inhibition of ADP-stimulated respiration by phenothiazines was best observed with succinate as substrate. In the presence of NADH-linked substrates, electron transport was inhibited by concentrations of these agents sufficient to inhibit ADP-stimulated respiration. This observation is illustrated in Fig. 2D, where the uncoupling agent FCCP was not able to stimulate respiration in mitochondria pretreated with 16 μM penfluridol while using pyruvate/malate as substrate. The further addition of succinate led to a stimulation of respiration, indicating that succinate bypassed a penfluridol-sensitive inhibition of electron transport.

Inhibition of ADP-stimulated respiration could have resulted from inhibition of either adenine nucleotide translocase or the oligomycin-sensitive ATPase. These two possibilities were distinguished by studying the distribution of labeled adenine nucleotide in the presence of calmodulin-binding agents. Mitochondria, incubated with [^{3}H]ADP in the presence of succinate exchanged external [^{3}H]ADP for internal ATP and phosphorylated the [^{3}H]ADP to form [^{3}H]ATP. Quantitation of the specific activity of the mitochondrial ADP pool was a measure of adenine nucleotide translocase activity, while quantitation of the ratio of mitochondrial [^{3}H]ATP/[^{3}H]-ADP was a measure of the activity of the oligomycin-sensitive ATPase. The addition of penfluridol (16 μM), pimozide (20 μM) or trifluoperazine (66 μM) prior to the addition of [^{3}H]ADP diminished the mitochondrial ATP pool in a manner analogous to oligomycin, but different from atractyloside (Table I). The loss of ATP from treated mitochondria probably resulted from exchange of external ADP for internal ATP with no subsequent regeneration of ATP. The expected increase in the ADP pool which should have resulted from this process, however, was not observed. Instead, phenothiazines reduced the ADP pool and the specific activity of the ADP pool by 38 and 6%, respectively (Table I), indicating that adenine nucleotide translocase was not affected. Atractyloside, which inhibits the adenine nucleotide translocase, produced a different pattern, reducing the ADP pool and specific activity of the ADP pool by 3 and 77%, respectively. All three phenothiazines decreased the ratio [^{3}H]ATP/[^{3}H]ADP in close agreement with the inhibition produced by oligomycin. Taken as a whole, the data indicated that the oligomycin-sensitive ATP-

TABLE I

MITOCHONDRIAL NUCLEOTIDE METABOLISM IN THE PRESENCE OF CALMODULIN-BINDING AGENTS

Sample	mol ATP/mg protein ($\times 10^9$)	mol ADP/mg protein ($\times 10^9$)	Specific activity of ADP pool (cpm/mol ADP) ($\times 10^{-12}$)	[^{3}H]ATP/[^{3}H]ADP
Control	4.2 ± 0.4	3.3 ± 0.8	3.5 ± 0.7	0.72 ± 0.2
Penfluridol	0.87 ± 0.2	1.2 ± 0.6	2.8 ± 0.7	0.29 ± 0.1
Pimozide	1.5 ± 0.6	2.6 ± 1.2	3.3 ± 0.5	0.18 ± 0.07
Trifluoperazine	1.6 ± 0.1	2.3 ± 0.4	3.8 ± 0.5	0.14 ± 0.02
Actractyloside	3.6 ± 0.1	3.2 ± 0.3	0.82 ± 0.1	0.35 ± 0.02
Oligomycin	1.45 ± 0.3	3.7 ± 0.6	3.3 ± 0.3	0.15 ± 0.01

ase had a component that was selectively inhibited by penfluridol, pimozide and trifluoperazine in proportion to the relative affinities of these compounds for calmodulin at a site insensitive to trifluoperazine sulfoxide.

The absence of a calmodulin requirement for ATPase inhibition

The use of intact mitochondria limited the possibility of evaluating whether or not calmodulin was involved in the interaction between phenothiazines and mitochondria. Consequently, inner membranes were prepared by either Lubrol treatment of mitoplasts [12] or sonication of intact mitochondria followed by centrifugation and passage through a cytochrome *c* affinity column [20]. Both procedures produced inside-out oriented membrane vesicles. The same results were obtained with vesicles obtained by either procedure. However, only the results obtained using vesicles prepared from Lubrol-treated inner membranes were described. As seen in Fig. 3A, the oligomycin-sensitive ATPase was very sensitive to penfluridol, pimozide and trifluoperazine (with K_i values of 2.0, 2.8 and 7.0 μM, respectively). By contrast, the addition of trifluoperazine sulfoxide (hydrophilic) or the hydrophobic, non-calmodulin-binding neuroleptic agent, Nebutal, produced little or no inhibition of the ATPase (Fig. 4). When calmodulin was added exogenously to the inner membrane preparation in amounts ranging from 0.01 to

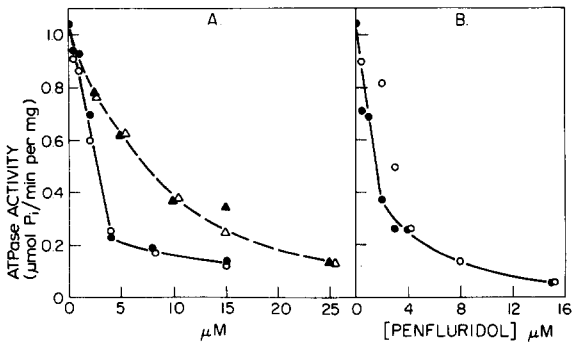

Fig. 3. The effects of penfluridol or trifluoperazine on ATPase activity of Lubrol-treated mitoplasts. Lubrol-treated mitoplasts were incubated as described in Materials and Methods, in the presence of penfluridol (●,○) or trifluoperazine (▲,△). The addition of Ca^{2+} (100 μM) (●,▲) or EGTA (10 mM) (○,△) preceded the addition of neuroleptic agent.

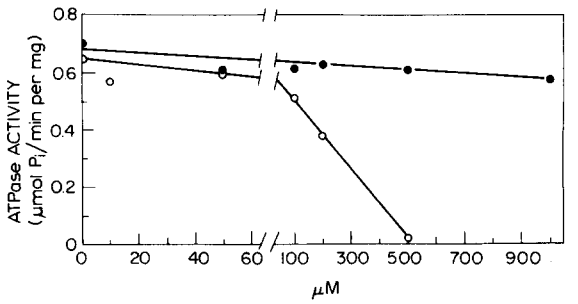

Fig. 4. The effect of Nebutal or trifluoperazine sulfoxide on the ATPase activity of Lubrol-treated mitoplasts. Nebutal (●) or trifluoperazine sulfoxide (○) was added in the presence of 100 μM Ca^{2+}. The inhibition of ATPase activity by trifluoperazine sulfoxide at concentrations greater than 100 μM may not be real, since trifluoperazine sulfoxdide interfered with the phosphate assay at these concentrations.

50 μg/ml, there was no effect on ATPase activity (Fig. 5). Such results are consistent with the notion that either calmodulin is already present in mitochondrial membranes, or does not participate in this system.

An important criterion for the use of phenothiazines as probes is that they should produce their effect only in the presence of Ca^{2+}. In the absence of Ca^{2+}, hydrophilic groups are exposed on the calmodulin, preventing phenothiazine binding. Therefore, it was significant that penfluridol, pimozide and trifluoperazine inhibited ATPase activity, both in the presence and absence of Ca^{2+} (Fig. 3A). The addition of 0.1–1 μM A23187 plus 1–10 mM EGTA did not

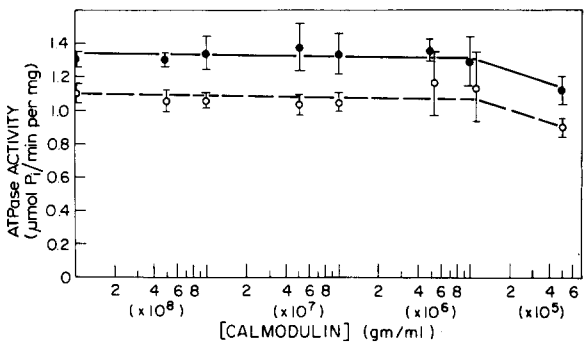

Fig. 5. The effects of calmodulin on ATPase activity of Lubrol-treated mitoplasts. ATPase activity was measured as described in Materials and Methods. Ca^{2+} (100 μM) (●) or EGTA (1 mM) (○) was added prior to the addition of bovine brain calmodulin.

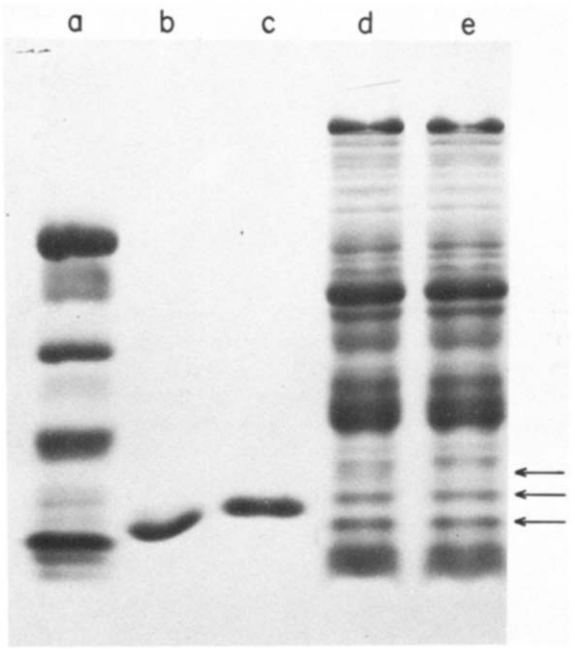

Fig. 6. Gel electrophoresis of calmodulin and Lubrol-treated mitoplasts on 7–15% gradient gels in the presence of SDS. (a) Molecular weight standards: bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A with M_r values of 67 000, 43 000, 25 000 and 13 700, respectively. (b) Bovine brain calmodulin plus 500 μM Ca^{2+}. (c) Bovine brain calmodulin plus 5 mM EGTA. (d) Lubrol-treated mitoplasts plus 500 μM Ca^{2+}. (e) Lubrol-treated mitoplasts plus 5 mM EGTA.

prevent the phenothiazine-induced inhibition of ATPase activity (Fig. 3B). This behavior indicated that the phenothiazine was binding to a hydrophobic group, the orientation of which was apparently not regulated by Ca^{2+}. In order to establish whether or not calmodulin was available in the inner membrane to bind phenothiazine, inner membrane preparations were layered over gradient polyacrylamide gels (7–15%) in the presence of SDS and in the presence of either Ca^{2+} or EGTA. As seen in Fig. 6, brain calmodulin exhibited a measurable shift in electrophoretic mobility under these conditions, but none of the mitochondrial proteins with a mobility similar to that of calmodulin underwent a comparable shift. Interestingly, an unknown mitochondrial protein with M_r of approx. 21 000 did exhibit a change in mobility in the presence of Ca^{2+}. In order to verify that calmodulin was not present in the inner membrane fraction, whole cells, intact mitochondria and mitochondrial inner membranes were assayed for their ability to activate brain cyclic nucleotide phosphodiesterase. As seen in Table II, calmodulin was detected in the cell homogenate and mitochondrial fraction, but was essentially absent from the inner membrane fraction. The data were not sufficient to establish whether the calmodulin in the mitochondrial fraction was derived solely from mitochondria or from redistribution of calmodulin during cell fractionation. The data, likewise, do not exclude the possibility that calmodulin plays a fundamental role in regulation of the oligomycin-sensitive ATPase. However, interaction of phenothiazines with the mitochondrial inner membrane occurs in the absence of calmodulin.

Despite the absence of calmodulin in the inner membrane preparation, [^{3}H]trifluoperazine bound to specific sites in the membrane (Fig. 7). Binding of [^{3}H]trifluoperazine to the preparation was completely prevented by the addition of a 100-fold excess of unlabeled trifluoperazine. Scatchard analysis of this binding (Fig. 7) revealed a single class of binding

TABLE II

DISTRIBUTION OF CALMODULIN IN RAT LIVER

Cell fraction	Specific calmodulin content (ng calmodulin/mg protein)	Total [a] (μg calmodulin/g liver)	% total in each compartment
Homogenate	232 ± 54	86	100
Mitochondria	34 ± 14	2.7	3
Lubrol-treated mitoplasts	0.27 ± 0.2	0.022	0.03

[a] The recovery of mitochondria and Lubrol-treated mitoplasts was determined by measuring succinate dehydrogenase activity [22]. In these experiments, 25% of the possible mitochondria and 20% of the inner membrane were recovered.

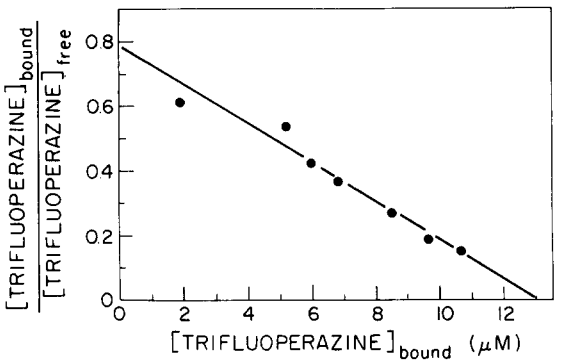

Fig. 7. Binding of [^{3}H]trifluoperazine to the Lubrol-treated mitoplasts. Mitochondrial inner membranes (100 μg/ml) were incubated with varying amounts of [^{3}H]trifluoperazine in the presence of 100 μM Ca^{2+} at pH 6.0 as described in Materials and Methods. The sample was filtered, washed and counted.

site with an association constant, K, of $6.5 \cdot 10^4$ M^{-1} (K_d = 15 μM), which is close to the reported affinity of trifluoperazine for calmodulin [5]. The amount of trifluoperazine bound per 100 μg of membrane protein was 12.5 nmol, corresponding to $7.5 \cdot 10^{16}$ binding sites. If all of this trifluoperazine were bound to calmodulin (assuming two binding sites per calmodulin molecule) there would have to be 100 μg calmodulin in the membrane sample. Since this corresponds to all of the protein in the inner membrane sample, trifluoperazine must have bound to components other than calmodulin.

Discussion

In spite of the fact that it is now generally accepted that Ca^{2+} has a nearly universal role in stimulus-response coupling [21], there is still a lack of detailed information as to its precise mechanisms of intracellular action. The discovery of the nearly universal distribution of calmodulin has led to the belief that it is a major, if not the major, intracellular calcium receptor protein. Hence, a study of its function in isolated enzyme and membrane systems is an area of much current research. In addition to studies with isolated enzymes, there is a need for methods which evalute the involvement of calmodulin in the control of metabolic events within intact tissues and cells. It has been proposed and widely accepted that phenothiazines are a valid probe for assessing the involvement of calmodulin in regulating cellular func- [4,23–25]. Such a use of phenothiazines assumes that they are specific metabolic inhibitors, the actions of which are confined to calmodulin-regulated events within the cell.

The present results indicate that the cellular effects of phenothiazines are less specific than believed, and that, therefore, some or many of their effects upon cell function may be mediated by their interaction with intracellular components other than calmodulin. Our results show that three different antipsychotic agents (penfluridol, pimozide and trifluoperazine) block ADP-dependent, but not Ca^{2+}-dependent or uncoupler-dependent respiration, in isolated rat liver mitochondria employing succinate as substrate. The drugs act by inhibiting the oligomycin-sensitive ATPase and do not influence the function of the adenine nucleotide translocase. A similar inhibition of site I respiration and mitochondrial ATPase activity by chlorpromazine had previously been reported by others [26]. Interestingly, a number of lipophilic cations, including octylguanidine [27] and triphenylsulphonium [28] produce the same inhibition of NADH-linked respiration and ATPase activity. Whether these compounds, like phenothiazines, also bind calmodulin has not been determined.

The concentration and relative potencies of phenothiazines required to produce the observed effects are similar to those which are needed to inhibit calmodulin-mediated enzymatic reactions (e.g., cyclic nucleotide phosphodiesterase). In spite of these similarities, studies with isolated vesicles prepared from inner mitochondrial membranes revealed that (a) the inhibitory action of the drugs was not influenced by changes in Ca^{2+} concentration or by exogenous calmodulin, (b) there was no detectable calmodulin in the vesicles and (c) the number of drug-binding sites was equivalent to the total amount of protein in the membrane preparations (assuming two binding sites per protein molecule and a molecular weight of 16 500). The binding data also raise the possibility that there are specific high-affinity binding sites in mitochondrial membrane lipids. Therefore, despite the specificity of the inhibition, phenothiazines disrupt mitochondrial ATPase activity by a calmodulin-independent reaction. We propose that phenothiazine-mediated alterations in cell function do not

necessarily implicate calmodulin as the regulator of that function.

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