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Effects of calmodulin antagonists on the active Ca^{2+} uptake by rat liver mitochondria

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The mechanism of Ca^{2+} transport by rat liver mitochondria was investigated with respect to the possible involvement of calmodulin in this process. We studied the action of exogenous calmodulin isolated from brain tissue on the Ca^{2+}-transport system, as well as the effect of two types of calmodulin antagonists; the phenothiazine drugs trifluoperazine and chlorpromazine and the more specific substance compound 48/80. Our results show that Ca^{2+} transport by mitochondria and mitochondrial ATPase activity are insensitive to exogenous calmodulin, although they can be inhibited by the phenothiazines. Since no effect of compound 48/80 was observed, we believe that the phenothiazines act through a mechanism that does not involve calmodulin. This is in accord with our inability to locate significant quantities of calmodulin in mitochondria by radioimmunoassay analysis. Our results further show that trifluoperazine and chlorpromazine also inhibit the electron-carrier system of the respiratory chain, and this effect may mediate their inhibitory action on Ca^{2+} transport when it is energized by respiration instead of ATP hydrolysis.

The low cytoplasmic Ca^{2+} concentration of living cells is maintained by energy-requiring pumps. These pumps either remove Ca^{2+} to the extracellular space by transport across the plasma membrane or accumulate it inside intracellular organelles such as mitochondria and endoplasmic reticulum (Borle, 1975; Carafoli *et al.*, 1975; Hasselbach, 1979; Sulakhe & Louis, 1980). Calmodulin, the multifunctional cellular Ca^{2+} receptor that regulates many Ca^{2+}-dependent processes, is also involved in the regulation of intracellular Ca^{2+} concentration (Vincenzi, 1979; Klee *et al.*, 1980; Marx, 1980). Indeed, calmodulin has been described as a modulator of the Ca^{2+} pump in erythrocyte membranes (Vincenzi & Larsen, 1980; Muallem & Karlish, 1981) and in cardiac sarcoplasmic reticulum (Katz & Remtulla, 1978; Le Peuch *et al.*, 1979; Lopaschuk *et al.*, 1980), but no effect on mitochondrial Ca^{2+} transport has been reported. The effect of calmodulin is inhibited by neuroleptic drugs, such as trifluoperazine and chlorpromazine, which have been utilized as useful tools to demonstrate the involvement of calmodulin in specific cellular functions (Weiss & Wallace, 1980). However, several authors have recently reported that the antipsychotic drugs of the phenothiazine group can affect various biological systems independently of their inhibition of the calmodulin function (Landry *et al.*, 1981; Corps *et al.*, 1982; Hirata *et al.*, 1982). Therefore, limitations must be considered concerning the use of these drugs as indicators of calmodulin-mediated processes. An inhibitor much more specific for calmodulin is compound 48/80, a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde, which was recently reported by Gietzen *et al.* (1983).

In the present work we studied the effect of chlorpromazine, trifluoperazine and compound 48/80 on the active Ca^{2+} uptake by rat liver mitochondria and we compared the effect of Ca^{2+} and exogenous calmodulin on ATPase activities of mitochondria, erythrocyte ghosts and sarcoplasmic reticulum. The results indicate that chlorpromazine and trifluoperazine inhibit active Ca^{2+} uptake and the respiratory activity of rat liver mitochondria by a process independent of calmodulin.

Experimental

Isolation of mitochondria

The mitochondria were isolated from rat liver by a modification of the method described by Bernard & Cockrell (1979). Livers were removed and homogenized in a solution containing 300 mM-mannitol, 5 mM-potassium glycylglycine and 0.1 mM-potassium EDTA at pH 7.4. After centrifugation for

10min at 800*g*, the supernatant was collected and centrifuged for 10min at 8000*g*. The isolated mitochondria were washed once in the same medium and, finally, they were resuspended in a medium containing 300mM-mannitol, 15mM-Tris/glycylglycine and 0.1mM-Tris/EDTA, pH 7.2.

Isolation of erythrocyte ghosts and sarcoplasmic reticulum

Erythrocyte ghosts were isolated from pig blood by the method of Buckley (1974). After haemolysis of the erythrocytes in 1mM-Tris/HCl and 1.4mM-EDTA (pH 7.4), the ghosts were collected by centrifugation for 15min at 10000*g* and then they were washed three times in a solution containing 1mM-Tris/HCl, 1.4mM-EDTA and 17mM-NaCl, pH 7.4. Finally, the white ghosts were washed twice with 20mM-Tris/HCl (pH 7.4) and were resuspended in this medium.

The sarcoplasmic-reticulum membranes were isolated from rabbit white skeletal muscle as reported previously (Vale & Carvalho, 1975).

The protein was determined by the biuret method with bovine serum albumin as standard (Layne, 1957).

Measurement of active Ca^{2+} uptake

Active Ca^{2+} uptake by mitochondria was carried out by using ATP or respiratory substrates as energy donors. The respiration-dependent process was performed in 5mM-Tris, 5mM-$MgCl_2$, 50mM-KCl, 6mM-succinate/glutamate, 80μM-$CaCl_2$ and 2mg of protein in a final volume of 2.5ml, at the pH value of 7.0. After 2min, the reaction was stopped by centrifugation in the cold for 10min at 8000*g*. A similar method was utilized to determine the ATP-dependent process, but the medium contained 2μg of antimycin and 8mM-ATP instead of respiratory substrates. In parallel experiments, the reaction was carried out in the presence of ATP plus substrates. The Ca^{2+} accumulated was determined in the pellets after extraction with a solution containing 2% trichloroacetic acid and 0.5% La^{3+}. The analysis was performed by atomic-absorption spectroscopy in a Perkin–Elmer spectrophotometer (model 305).

In some experiments, the mitochondria were first washed with 1mM-EGTA and then they were used for analysis of Ca^{2+} transport.

Measurement of ATPase activities

The mitochondrial ATPase activity was determined in the medium described above for the analysis of Ca^{2+} uptake. The reaction was started by adding 8mM-ATP and after 2min it was stopped by adding cold trichloroacetic acid to a final concentration of 7.5%. After centrifugation for 10min at 6000*g*, P_i analysis was performed by the method of Taussky & Shorr (1953).

In some experiments, ATPase activity was determined by measuring the H^+ production associated with the ATP hydrolysis (Nishimura *et al.*, 1962; Martonosi & Feretos, 1964; Deamer, 1973). The reaction medium contained 5mM-Tris/HCl, 5mM-$MgCl_2$, 50mM-KCl, 0.2mM-EGTA, 0.3mM-$CaCl_2$, 2μg of antimycin, 20μM-X-537A (if present), 0.5–1.0mg of protein and 1mM-ATP in a final volume of 5ml at pH 7.0.

Radioimmunoassay for calmodulin

The determination of the mitochondrial calmodulin was performed by the NEN radioimmunoassay method in which a radioactive and non-radioactive antigen compete for the same antibody-binding sites. Mitochondrial preparations containing 100mg of protein were previously heated for 7min at 87°C and then they were centrifuged for 10min. Portions (100μl) of the supernatant were allowed to react with a calmodulin antibody for 18h at 4°C in the presence of exogenous ^{125}I-calmodulin. The assay medium contained 0.125M-borate (pH 8.4), 0.075M-NaCl, 0.2% bovine serum albumin, 1mM-EGTA and 0.1% NaN_3. After the incubation period, the antigen–antibody complexes were separated from free antigen by addition of an accelerated second antibody system. After 30min of reaction at 4°C, the suspensions were centrifuged for 15min at 5000*g*, and the radioactivity was measured in the pellets by a Packard gamma scintillation counter. The amount of mitochondrial calmodulin was determined from a standard curve of bovine brain calmodulin using the manufacturer's (New England Nuclear) instructions.

Assay of mitochondrial respiration

The respiratory activity of the mitochondria was determined by measuring the O_2 consumption with a Clarke-type oxygen electrode. The reaction medium contained 300mM-mannitol, 10mM-Tris, 10mM-KCl, 5mM-potassium phosphate, 0.1mM-potassium EDTA, 11mM-succinate and 4mg of mitochondrial protein in a final volume of 4ml, at pH 7.2. The oxidation–phosphorylation coupling was observed by adding 700nmol of ADP. In some experiments, uncoupling was obtained by adding 25μM-dinitrophenol.

Materials

All reagents were analytical grade. Trifluoperazine was obtained from Smith Kline and French. Chlorpromazine and compound 48/80 were supplied by Sigma Chemical Company and calmodulin ^{125}I radioimmunoassay kit was obtained from New England Nuclear. The ionophore X-537A was a gift from Dr. Julius Berger of Hoffman–La Roche, Nutley, NJ, U.S.A., and brain calmodulin was

generously supplied by Dr. João Alface, Department of Zoology, University of Coimbra.

Results and discussion

Effect of chlorpromazine and trifluoperazine on active Ca^{2+} uptake and ATPase activity of rat liver mitochondria

Mitochondria can transport Ca^{2+} by using the energy of respiration or of ATP hydrolysis. Fig. 1 shows that the phenothiazine drugs chlorpromazine or trifluoperazine inhibit the ATP-dependent and the respiration-dependent Ca^{2+} uptake in a concentration-dependent manner. However, the effect of trifluoperazine is more potent that that of chlorpromazine, especially at concentrations higher than 80μM. In the presence of ATP, higher amounts of Ca^{2+} were accumulated by mitochondria, probably because the nucleotide facilitates the retention of Ca^{2+} within these organelles (Harris, 1979). The lower amounts of Ca^{2+} accumulated in the presence of phenothiazines probably are not due to disruption of the membrane, since it has been reported recently that these drugs increase the stability of the membranes (Landry *et al.*, 1981) and reduce the mitochondrial Ca^{2+} efflux (Harris & Cooper, 1982).

Measurements of ATPase activity, determined in the presence of antimycin, show that the ATP hydrolysis is reduced to about 50% by 200μM of either trifluoperazine or chlorpromazine (Fig. 2). This effect is not evident when mitochondrial respiration is operative, since, under these conditions, the amount of ATP hydrolysed is very low (Fig. 2). It appears that the respiratory chain in itself has some regulatory role on the ATPase activity, as was previously observed by Tu *et al.* (1981).

Since trifluoperazine and chlorpromazine have been described as calmodulin antagonists, we investigated whether the inhibitor effect of these drugs on mitochondrial Ca^{2+} uptake is mediated by inactivation of calmodulin. We therefore investigated the effect of a more specific calmodulin antagonist, compound 48/80, as well as the action of exogenous calmodulin on Ca^{2+} transport by mitochondria. Fig. 3 shows that a concentration of compound 48/80 (40μg/ml) that completely inhibits the stimulatory effect of calmodulin on Ca^{2+}-dependent ATPase of erythrocyte membranes (Fig. 3*b*) has no effect either on the Ca^{2+} transport or on the ATPase activity of the mitochondria (Fig. 3*a*). We also observed that mitochondrial Ca^{2+} transport is insensitive to exogenous calmodulin.

Fig. 4 shows that ATP-dependent Ca^{2+} uptake by mitochondria occurs at equal rates in EGTA-washed or non-washed mitochondria, and that the addition of calmodulin, isolated from bovine brain, does not alter the rate of Ca^{2+} uptake as determined by measuring the H^+ liberation associated with the active Ca^{2+} transport. The lack of effect of exogenous calmodulin on the response of mitochondria to Ca^{2+} is not due to the presence of intrinsic calmodulin, since similar results were obtained utilizing either intact mitochondria or mitochondria previously washed with EGTA, which should remove the modulator protein (Kakiuchi *et al.*, 1978).

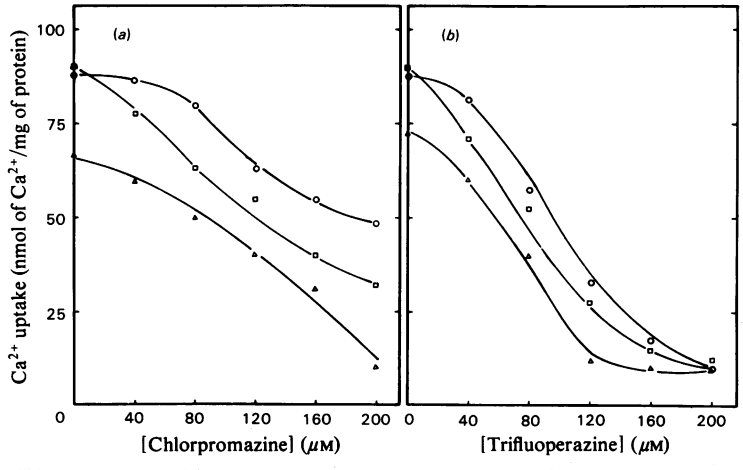

Fig. 1. *Effect of chlorpromazine (a) and trifluoperazine (b) on active Ca^{2+} uptake by rat liver mitochondria*
The experiments were carried out in a medium containing 5mM-Tris, 5mM-$MgCl_2$, 50mM-KCl, 6mM-succinate/glutamate, 80μM-$CaCl_2$ and 2mg of protein in a final volume of 2.5ml at pH 7.0. After 2min, the reaction was stopped by centrifuging for 8min in the cold. In some experiments, the reaction was carried out in the presence of respiratory substrates plus 8mM-ATP, or in the presence of 8mM-ATP and 2μg of antimycin. Ca^{2+} uptake was measured in the presence of: △, respiratory substrates; ○, ATP; □, ATP plus respiratory substrates.

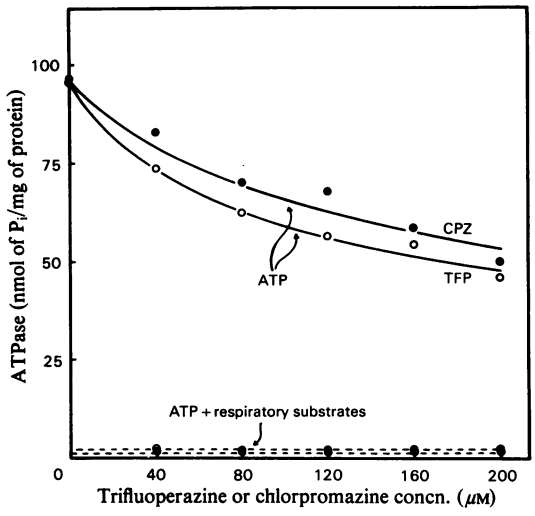

Fig. 2. *Effect of chlorpromazine and trifluoperazine on the ATPase activity of rat liver mitochondria*

The mitochondrial ATPase activity was determined in a medium as described in the legend to Fig. 1. The reaction was started by adding 8mM-ATP and after 2min it was stopped by adding 7.5% trichloroacetic acid. The analysis of P_i was performed as described in the text. ● and ○, ATPase activity in the presence of chlorpromazine (CPZ) or trifluoperazine (TFP) respectively. The broken lines represent the ATPase activity in the presence of respiratory substrates.

These results are in accord with our inability to locate significant amounts of calmodulin in rat liver mitochondria, isolated under the conditions described here. By using a radioimmunoassay method,

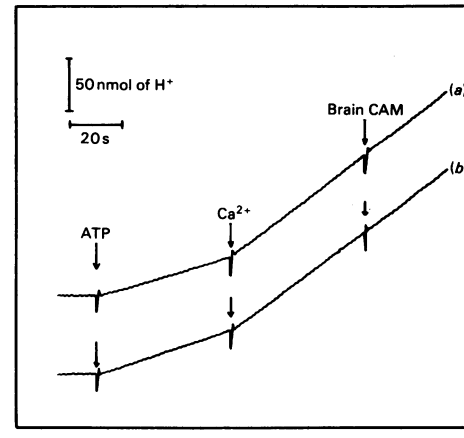

Fig. 4. *Effect of exogenous calmodulin on active Ca^{2+} uptake by mitochondria*

(*a*) The reaction was followed by measuring the H^+ liberation in a medium (5ml) containing 5mM-Tris, 5mM-$MgCl_2$, 50mM-KCl, 1mM-ATP, 50μM-$CaCl_2$ and 1mg of protein at pH7.0. Brain calmodulin (80μg; CAM) was added 50s after the Ca^{2+} addition. (*b*) In parallel experiments, the mitochondria were previously washed with 1mM-EGTA.

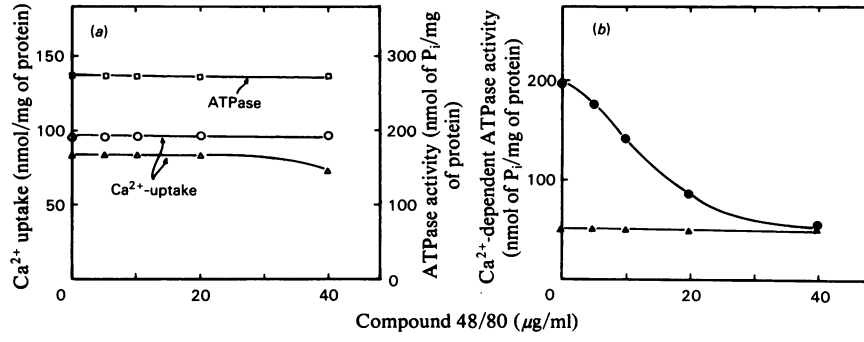

Fig. 3. *Effect of compound 48/80 on active Ca^{2+} transport by mitochondria (a) and on Ca^{2+}-dependent ATPase activity of erythrocyte membranes (b)*

Mitochondrial Ca^{2+} uptake and ATPase activity were determined as described in the text. The Ca^{2+}-dependent ATPase activity of erythrocyte ghosts was measured in the absence and in the presence of 50μg of calmodulin isolated from bovine brain. The reaction was started by adding 5mM-ATP, and 5min later it was stopped by adding 7.5% trichloroacetic acid. The analysis of P_i liberated and Ca^{2+} accumulated were performed as described in the text. △, Respiration-dependent Ca^{2+} uptake; ○, ATP-dependent Ca^{2+} uptake; □, ATPase activity by mitochondria; ▲, basal Ca^{2+}-dependent ATPase activity; ●, calmodulin-stimulated Ca^{2+}-dependent ATPase activity by erythrocyte ghosts.

we found only 1.5ng of calmodulin per mg of mitochondrial protein. This amount of calmodulin corresponds to the fraction of intramitochondrial calmodulin (20%) recently isolated by Hatase *et al.* (1982). The value extrapolated from their results (approx. 1.4ng/mg of mitochondrial protein) is quite similar to that obtained for our preparations (approx. 1.5ng/mg of mitochondrial protein). The other fraction of calmodulin (80%) that they obtained was of cytosolic origin. Presumably, this fraction of calmodulin was not present in our preparations, since we carried out all isolation procedures in the presence of EDTA. On the other hand, the assumption should not be made that mitochondrial intrinsic calmodulin (approx. 1.5ng/mg of protein) is high enough to support Ca^{2+} transport since much higher amounts of calmodulin (approx. 10μg/mg of protein) have been demonstrated to be necessary for stimulation of other Ca^{2+}-pump systems (Katz & Remtulla, 1978; Alface & Pires, 1982).

These findings indicate that calmodulin is not directly involved in the mechanism of Ca^{2+} transport by rat liver mitochondria, as was also previously observed in plant mitochondria (Dieter & Marmé, 1980) and in mitochondria of guinea-pig macrophages (Hirata *et al.*, 1982). The lack of effect of calmodulin on ATP-dependent Ca^{2+} transport by mitochondria is consistent with the observation that ATPase activity, measured in the presence of ionophore X-537A, is not stimulated by Ca^{2+}, which significantly increases the ATPase activity of the sarcoplasmic reticulum under similar conditions (Fig. 5). Mitochondrial ATPase actually is partially inhibited by Ca^{2+}, which suggests that, in contrast with the reticulum membranes, the mitochondrial ATPase is not directly responsible for the transport of Ca^{2+}. A Ca^{2+} carrier of the mitochondria has been reported to be a glycoprotein (Panfili *et al.*, 1976), but the energy of respiration or of ATP hydrolysis is utilized to generate a membrane potential (and a proton gradient) that is responsible for driving Ca^{2+} in electrophoretically (Rottenberg & Scarpa, 1974). Our results show that Ca^{2+} transport by mitochondria and mitochondrial ATPase activity are insensitive to exogenous calmodulin although they can be inhibited by phenothiazines. Since no effect of compound 48/80 was observed, we believe that the phenothiazines act through a mechanism that does not involve calmodulin. This assumption is supported by the results of the experiments described below.

Effect of chlorpromazine and trifluoperazine on mitochondrial respiration

The effect of chlorpromazine and trifluoperazine on the respiratory activity of mitochondria was determined by measuring the rate of O_2 consumption in the presence of ADP or dinitrophenol, using succinate as the electron donor. Fig. 6 shows that chlorpromazine or trifluoperazine inhibits mitochondrial respiration in a concentration-dependent manner, and that the effect of trifluoperazine is more potent than that of chlorpromazine. The sensitivity to the drugs, which is particularly evident in the case of chlorpromazine, is higher when the substrate oxidation is coupled to phosphorylation than when the system was first uncoupled by adding dinitrophenol (Fig. 6*a*). Thus the effect of the drugs strictly on the electron carriers of the respiratory chain is different from that measured simultaneously on the phosphorylation machinery, which involves the ATPase system. The electron carriers alone (dinitrophenol present) are affected at concentrations only above 120μM-chlorpromazine, whereas ADP-stimulated respiration is inhibited at concentrations as low as 40μM-chlorpromazine (Fig. 6*a*).

Calculations of the ADP/O ratio give a value of about 2 for concentrations up to 80μM-chlorpromazine or -trifluoperazine, whereas above this concentration we obtained values higher than 2 (Table 1). These results indicate that the drugs alter the rate of the oxidative-phosphorylation process, but not its efficiency. The high values obtained at high concentrations of drugs (Table 1) probably reflect direct effects on the mitochondrial membranes.

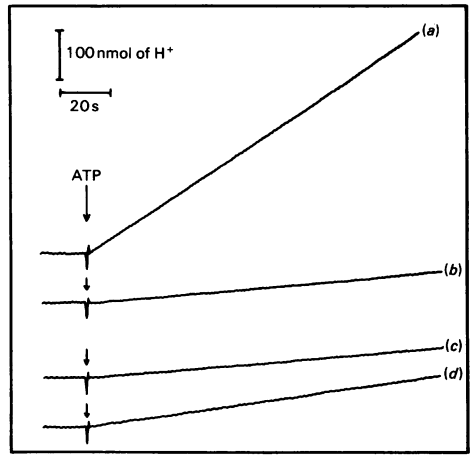

Fig. 5. *Comparison between the effect of Ca^{2+} on ATPase activity of sarcoplasmic reticulum (a and b) and of mitochondria (c and d)*

The reaction was carried out as described in the legend to Fig. 4, except that 20μM-X-537A, 2μg of antimycin, 0.2mM-EGTA and 0.3mM-$CaCl_2$ (if present) were also added to the medium. The reaction was started by adding 1mM-ATP. (*a*) and (*c*), Ca^{2+} present; (*b*) and (*d*), Ca^{2+} absent.

Table 1. *Effect of chlorpromazine and trifluoperazine on the ADP/O ratio of rat liver mitochondria*
The experiments were performed as described in the legend to Fig. 6.

Drugs ...	Chlorpromazine					Trifluoperazine				
Concn. (μM) ...	0	40	80	120	160	0	40	60	80	100
ADP/O	2.1	2.1	2.0	2.2	3.5	2.2	2.1	1.9	2.1	3.2

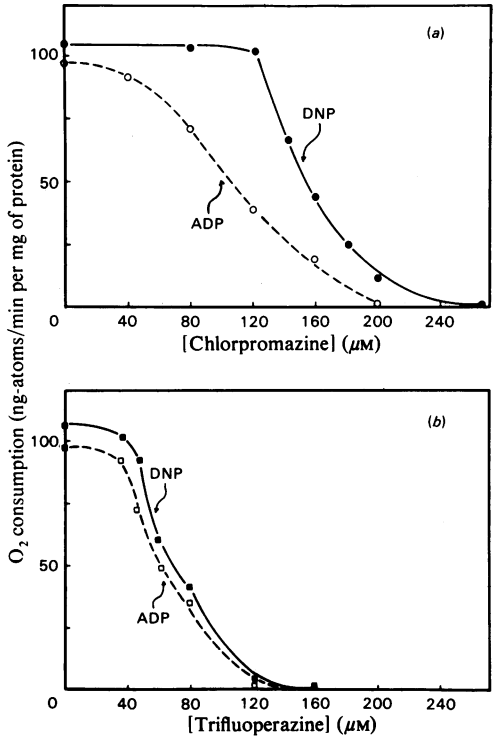

Fig. 6. *Effect of chlorpromazine (a) and trifluoperazine (b) on the respiratory activity of rat liver mitochondria*
The reaction medium (4 ml) contained 300 mM-mannitol, 10 mM-Tris, 10 mM-KCl, 5 mM-potassium phosphate, 0.1 mM-potassium EDTA, 11 mM-succinate and 4 mg of mitochondrial protein at pH 7.2. In one group of experiments, chlorpromazine or trifluoperazine was added before ADP (700 nmol), and in another group, the drugs were added after uncoupling the oxidative phosphorylation with 25 μM-dinitrophenol (DNP). The O_2 consumption was monitored with a Clark-type oxygen electrode connected to a Vitatron recorder. ○, ADP-stimulated respiration; ●, dinitrophenol-uncoupled respiration.

These findings are in agreement with those of Ruben & Rasmussen (1981), who observed that phenothiazines and related compounds disrupt mitochondrial energy production by a reaction that apparently does not involve a drug–calmodulin interaction.

The results reported in the present paper indicate that the inhibitory effect of chlorpromazine and trifluoperazine on the respiration-dependent and ATP-dependent Ca^{2+} uptake by rat liver mitochondria is due to direct action of the drugs on the electron carriers and on the ATPase molecule, rather than on calmodulin. These observations, in agreement with those reported by other investigators (Landry *et al.*, 1981; Corps *et al.*, 1982), limit the use of antipsychotic drugs as indicators of calmodulin-mediated processes.

In conclusion, calmodulin appears to be not directly involved in the mechanism of Ca^{2+} transport by mitochondria. However, as was reported by Reinhart *et al.* (1980), calmodulin seems to be involved in the phenylephrine-induced Ca^{2+} uptake by mitochondria, which suggests that the regulator protein controls the mechanism of action of the α-adrenergic agonists and, indirectly, mitochondrial Ca^{2+} fluxes in liver.

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