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Mechanism of Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase by Tricyclic Antipsychotics

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SUMMARY

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The interaction of the thioxanthene derivative flupenthixol and its phenothiazine analogue, fluphenazine, with brain (Na^+ + K^+)-ATPase [Mg^{++}-dependent, (Na^+-K^+)-activated ATP phosphohydrolase, EC. 3.6.1.3] and the associated potassium-dependent *p*-nitrophenyl phosphatase (EC. 9.6.1.7) was studied under conditions preventing formation of semiquinone free radicals of the drugs. Inhibition of (Na^+ + K^+)-ATPase is enhanced by Mg·ATP and antagonized by both sodium and potassium. The influence of pH indicates that both drugs are more effective in the unprotonated, lipophilic form. The data on formation of ^{32}P-labeled enzyme suggest that phosphorylation of the enzymatic protein is affected by the two drugs. Inhibition of K^+-dependent *p*-nitrophenyl phosphatase is competitively antagonized by potassium. It is proposed that flupenthixol and fluphenazine bind to the enzyme-substrate complex, preventing subsequent activation by sodium and potassium. No difference was recorded between the thioxanthene and the phenothiazine derivative.

INTRODUCTION

The ability of tricyclic antipsychotics to interact with membranes has been widely documented. Alteration of membrane permeability and inhibition of transport phenomena have been proposed as possible mechanisms explaining their central effects (1). Since it was recognized that (Na^+ + K^+)-ATPase is involved in the active transport of sodium and potassium (2), much attention has been focused on the inhibitory effect of antipsychotics on this key enzyme system. A correlation was shown to exist between the inhibitory potency of various substituted phenothiazines and their antipsychotic activity (3). The electron donor ability (4-7) or the hydrophobic character (8) of these drugs was proposed to be important in the interaction with the enzyme.

Detailed information is still lacking on the biochemical effects of the chemically and pharmacologically related thioxanthene derivatives. The ability of chlorprothixene to inhibit (Na^+ + K^+)-ATPase *in vitro* was first reported by Ebadi and Carver (9), but the nature of the interaction of thioxanthenes with (Na^+ + K^+)-ATPase was not characterized further.

This paper describes the molecular interaction of flupenthixol, a potent piperazinyl thioxanthene derivative, with (Na^+ + K^+)-ATPase obtained from guinea pig brain. Identical experiments with fluphen-

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azine, the phenothiazine analogue of flupenthixol, were also performed. To obtain more detailed information, analysis of the effects of the drugs was extended to potassium-dependent *p*-nitrophenyl phosphatase, which is believed to represent a partial reaction of the $(Na^+ + K^+)$-ATPase system (10, 11).

METHODS

Enzyme preparation. ATPase-containing microsomes were prepared from guinea pig brain as described by Hokin and Hokin (12). The microsomal fraction was subsequently treated with NaI according to Uesugi *et al.* (13).

Enzyme assays. ATPase activity, unless otherwise stated, was tested at 37° in the following incubation medium: 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP-Tris (pH 7.4), 50 mM Tris-HCl (pH 7.4), 50 mM sucrose, 0.2 mM EDTA, and 40–80 μg of enzyme preparation in a final volume of 1 ml. The incubation time was 10 min. The reaction was started by the addition of enzyme and stopped by the addition of 0.25 ml of cold 50% trichloracetic acid. Inorganic phosphate was determined according to Fiske and SubbaRow (14).

The $(Na^+ + K^+)$-ATPase activity (60–100 μmoles of P_i per milligram of protein per hour) was taken as the difference between the total activity and the activity in the presence of 0.2 mM ouabain. K^+-activated *p*-nitrophenyl phosphatase activity was assayed at 37°, using the incubation medium described by Robinson (15). The incubation time was 10 min.

Spectrophotometric determination of ATPase activity. $(Na^+ + K^+)$-ATPase activity was assayed by continuously measuring the oxidation of NADH in an Eppendorf spectrophotometer at 366 nm, using the procedure of Wallick *et al.* (16).

^{32}P incorporation procedure. The labeling reaction was carried out at 0° in duplicate tubes following the procedure of Erdmann and Schoner (17), except that 5 mM unlabeled ATP and 1 mM KH_2PO_4 were present in the solutions of 5% trichloracetic acid used to stop the reaction and wash the precipitate.

Protein was determined as described by Lowry *et al.* (18), using crystalline bovine serum albumin as a standard.

Materials. ATP, *p*-nitrophenyl phosphate, bovine serum albumin, NADH, and phosphoenolpyruvate were obtained from Sigma, and pyruvate kinase and lactate dehydrogenase, from Boehringer/Mannheim. Flupenthixol and fluphenazine hydrochloride were generous gifts from Carlo Erba (Milan) and Squibb (Rome), respectively.

Terminally labeled [^{32}P]ATP was obtained from the Radiochemical Centre.

RESULTS

The occurrence of semiquinone free radicals when solutions of tricyclic antipsychotics are exposed to light makes it difficult to discriminate between the inhibition induced by the free radical and by the drug itself (4). In preliminary experiments it was noticed that inhibition was not constant when the enzymatic reaction was performed in daylight, suggesting variable participation of free radicals. To prevent free radical formation, therefore, all experiments were performed in the dark with fresh solutions of drugs. The residual Mg^{++}-dependent, ouabain-insensitive ATPase activity (5–15% of the total activity) was not inhibited significantly by flupenthixol and fluphenazine under these experimental conditions.

Reversibility of inhibition of $(Na^+ + K^+)$-ATPase by flupenthixol and fluphenazine. Figure 1 shows the time course of $(Na^+ + K^+)$-ATPase inhibition by flupenthixol and fluphenazine. With both drugs inhibition was independent of time, indicating that true equilibrium was readily attained. This behavior is suggestive of a reversible interaction between the enzyme and the drugs (19). In separate experiments reversibility was also observed after incubation of the enzyme complex with drugs and subsequent washing.

As the free radical of chlorpromazine has been shown to bind irreversibly to essential free sulfhydryl groups of $(Na^+ + K^+)$-ATPase (6), reversibility indicated that under such conditions free radical formation did not occur. No protection against the inhibitory effects of 0.1 mM flupenthixol or 0.2 mM fluphenazine was afforded by either dithiothreitol or cys-

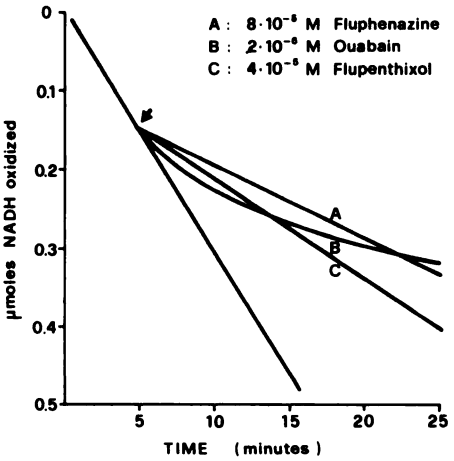

FIG. 1. *Continuous spectrophotometric recording of ATPase activity*

Each cuvette contained 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 3 mM ATP-Tris, 50 mM Tris-HCl (pH 7.4), 50 mM sucrose, 0.2 mM EDTA, 2 mM phosphoenolpyruvate, 0.5 mM NADH, 30 µg of pyruvate kinase, 15 µg of lactate dehydrogenase, and 13 µg of enzyme preparation in a final volume of 1 ml at 37°. Inhibitors were added at the time shown by the arrow, in a volume of 0.01 ml. An equal volume of water was added to the control cuvette. Flupenthixol and fluphenazine did not affect pyruvate kinase and lactate dehydrogenase activities at the concentrations used. The inhibition pattern of ouabain, a pseudoirreversible inhibitor (16), is shown for comparison.

teine up to 300 µM in the incubation mixture. Reversibility of inhibition of $(Na^+ + K^+)$-ATPase by chlorpromazine (incubation performed in the dark) has recently been reported by Roufogalis (8).

Effect on $(Na^+ + K^+)$-ATPase and K^+-p-nitrophenyl phosphatase activities. From dose-response curves the concentrations of flupenthixol required for 50% inhibition (I_{50}) of $(Na^+ + K^+)$-ATPase and K^+-p-nitrophenyl phosphatase were determined to be 80 and 70 µM, respectively. Fluphenazine was less effective in both cases, with I_{50} values of 160 µM for $(Na^+ + K^+)$-ATPase and 100 µM for K^+-p-nitrophenyl phosphatase. The greater sensitivity of the latter activity may be due in part to the higher pH of the incubation medium: pH 7.8 for the phosphatase compared with pH 7.4 for $(Na^+ + K^+)$-ATPase. Figure 2 shows that, under conditions which do not allow free radical formation, the effectiveness of flupenthixol and fluphenazine as inhibitors of $(Na^+ + K^+)$-ATPase was a function of increasing pH. This suggests that the unprotonated, lipid-soluble forms of these drugs are the active species interacting with the enzyme.

Effect of Mg·ATP. The effects of flupenthixol and fluphenazine on the kinetics of ATP hydrolysis are shown in Fig. 3 as double-reciprocal plots. A Mg:ATP concentration ratio of 1 was maintained in all experiments. Concentrations of the Mg·ATP complex were calculated assuming a value of 10^4 M^{-1} for the stability constant (20). It should be noted that if the stability constant of the Mg·ATP complex is assumed to be infinite (21), indication of positive cooperativity is obtained, in agreement with Squires (22). A slight tendency for deviation from linearity is still detectable in Fig. 3. Both drugs caused a parallel upward shift of the control line, typical of uncompetitive inhibition. Inhibition of $(Na^+ + K^+)$-ATPase activity by flu-

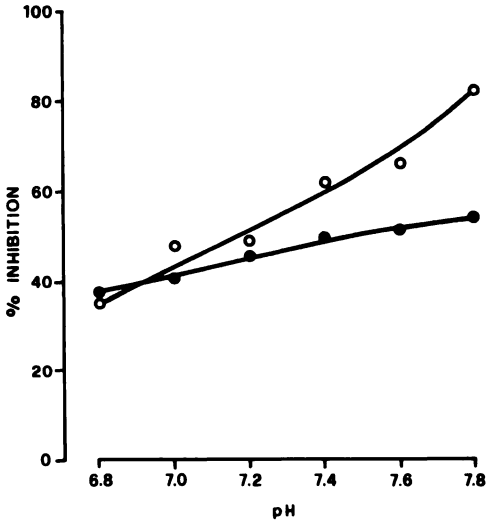

FIG. 2. *pH dependence of inhibition of $(Na^+ + K^+)$-ATPase by flupenthixol and fluphenazine*

The incubation media were buffered with 50 mM Tris-HCl at the required pH. The other conditions were the same as described in METHODS. $(Na^+ + K^+)$-ATPase activity varied from 53 (pH 6.8) to a maximum of 62 µmoles/mg/hr (pH 7.4). Inhibition values were calculated from controls at each pH. Each point represents the mean of three experiments. ●——●, 0.1 mM flupenthixol; ○——○, 0.2 mM fluphenazine.

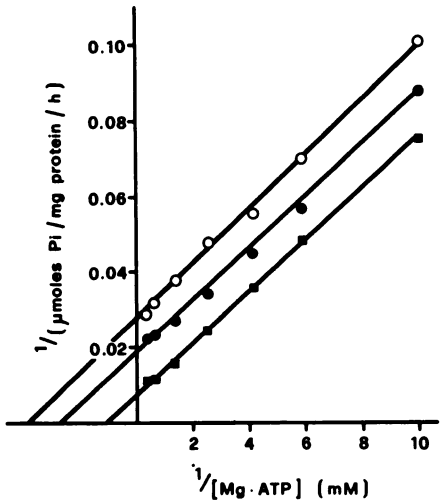

FIG. 3. *Effects of flupenthixol and fluphenazine on kinetics of ATP hydrolysis*

Experimental conditions were the same as described in METHODS, except that ATP and $MgCl_2$ were varied, in equimolar concentrations, from 0.2 to 3 mM. Each point represents the mean of three separate experiments. ■——■, control; ●——●, .08 mM flupenthixol; ○——○, 0.2 mM fluphenazine.

penthixol increased from 11%, at 0.2 mM ATP, to 59%, at 3 mM ATP. With fluphenazine the increase in ATP concentration from 0.2 to 3 mM was accompanied by an increase in inhibition from 28% to 61%.

To exclude a possible effect of increasing concentration of ADP generated by ATP hydrolysis, identical experiments were performed in the presence of an ATP-regenerating system consisting of 2 μmoles of phosphoenolpyruvate and 30 μg of pyruvate kinase. Control experiments showed that the drugs, in the amounts present in the assay medium, did not decrease the efficiency of the regenerating system. Neither the kinetic behavior of the $(Na^+ + K^+)$-ATPase reaction nor the inhibition pattern of the drugs was modified by the regenerating system.

Effects of various sodium and potassium concentrations. As pointed out by Priestland and Whittam (23), kinetic analysis of cation activation of $(Na^+ + K^+)$-ATPase using fragmented membrane preparations is difficult to interpret because of the possible competition between sodium and potassium for the sites of activation. It seemed convenient, therefore, to plot the data according to the Hunter-Downs method, which does not require extrapolation of the regression lines but permits direct discrimination of the type of inhibition from the shape of the line: a line with a positive slope is indicative of competitive inhibition, whereas noncompetitive inhibition would give a line with a slope of zero, i.e., parallel to the abscissa (19). The effects of increasing concentrations of sodium and potassium on inhibition of $(Na^+ + K^+)$-ATPase by flupenthixol and fluphenazine are shown in Figs. 4 and 5. Both Hunter-Downs plots yielded lines with downward curvature. The slope of the curves remained positive throughout the concentration range of the activating ions, however, suggesting that inhibition was competitive with respect to both sodium and potassium. On the other hand, apparent noncompetitiveness at the higher concentrations of sodium or potassium is to be expected for any drug competing with both

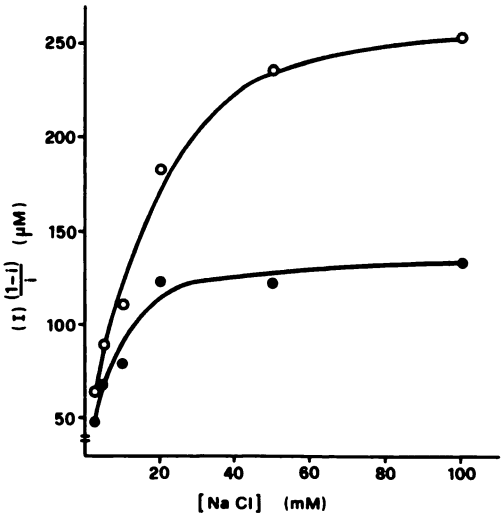

FIG. 4. *Hunter-Downs plot: effect of sodium*

(I) represents the concentration of flupenthixol or fluphenazine, and i is the fractional inhibition. The potassium concentration was kept constant at 20 mM. Choline chloride was used to maintain constant ionic strength. Exclusion of choline chloride, however, had no effect on the results. The other experimental conditions were the same as described in METHODS. Each point represents the mean of three experiments. ●——●, .08 mM flupenthixol; ○——○, 0.16 mM fluphenazine.

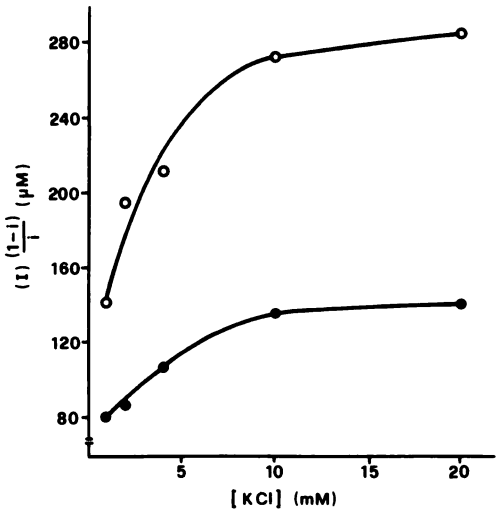

FIG. 5. *Hunter-Downs plot: effect of potassium*
Symbols and concentrations of inhibitors are described in the legend to Fig. 4. The sodium concentration was kept constant at 100 mM. The other experimental conditions were the same as described in METHODS. Each point represents the mean of three experiments.

activators (24), as increasing the concentration of an ion cannot overcome the inhibition of activation by the other ion.

Figure 6A shows that, in agreement with previous findings (15), the activation of *p*-nitrophenyl phosphatase by potassium was a cooperative phenomenon. V_{max} remained unchanged in the presence of both flupenthixol and fluphenazine, whereas the degree of cooperativity, reflected by the upward curvature of the lines, was increased. The latter effect can be better appreciated in Fig. 6B, where the same data are replotted in the Hill form. The Hill coefficient for potassium and the $K_{0.5}$ value (activator concentration at half-maximal velocity) were enhanced by the two drugs. The kinetic parameters for potassium activation in the presence and absence of the drugs are given in Table 1.

Formation and breakdown of ^{32}*P-labeled enzyme.* Table 2 shows the effect of flupenthixol and fluphenazine on sodium-dependent phosphorylation of $(Na^+ + K^+)$-ATPase. Formation of ^{32}P-labeled enzyme was much less sensitive than the total ATPase reaction. However, when the effect of flupenthixol and fluphenazine on $(Na^+ + K^+)$-ATPase was tested in an incubation medium containing 0.5 mg of enzyme preparation and only 0.5 mM ATP, the sensitivity of the total ATPase reaction was also greatly reduced. This indicated that the inhibition of sodium-activated phosphorylation by flupenthixol and fluphenazine was partially masked by the experimental conditions needed for the labeling reaction.

Table 3 shows the effects of increasing concentrations of flupenthixol and fluphenazine on the steady-state concentration of the phosphorylated intermediate in the presence of both sodium and potassium. The steady-state level of ^{32}P-labeled enzyme was decreased to the same extent as in the presence of sodium alone, suggesting that only phosphorylation was affected. Separate experiments, in which potassium chloride was added after 15 sec, gave essentially the same results.

DISCUSSION

Since 1959, when Karreman *et al.* (25) proposed the hypothesis that the therapeutic action of chlorpromazine may be due to its charge-transfer properties, the biological significance of the electron donor ability of tricyclic antipsychotic agents has been widely investigated. Akera and Brody (4–7) demonstrated that the semiquinone free radical of phenothiazine derivatives is a potent inhibitor of microsomal $(Na^+ + K^+)$-ATPase and proposed that the free radical, rather than the phenothiazines themselves, is responsible for the inhibition of $(Na^+ + K^+)$-ATPase and the effect *in vivo*. However, recent investigations (26–31) on electron donor power and free radical stability of many tricyclic antipsychotic agents indicated that this might not be the only explanation, since no correlation was found between free radical stability and antipsychotic activity (see also ref. 32).

This study, showing that tricyclic antipsychotics can inhibit $(Na^+ + K^+)$-ATPase even under conditions which do not allow free radical formation, demonstrates that the unmodified molecules of these drugs are also effective in inhibiting the enzyme. The concentrations of unmodified drugs

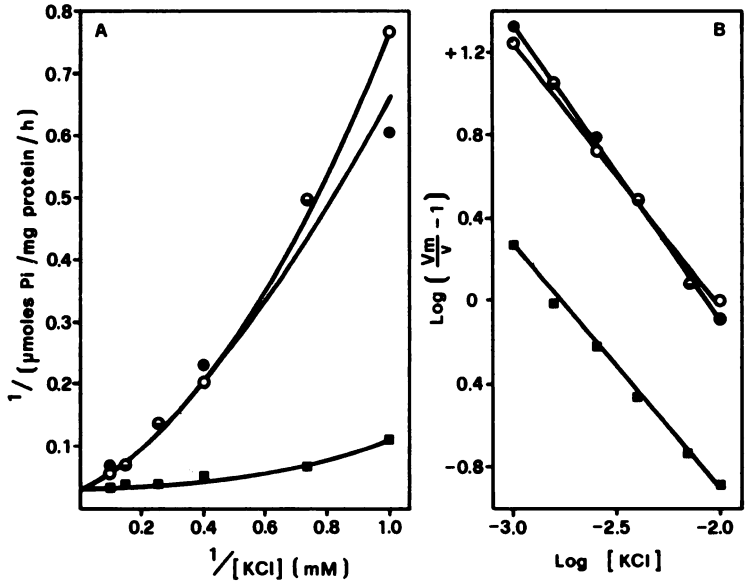

FIG. 6. *Effect of potassium on inhibition of K+-dependent p-nitrophenyl phosphatase*

A. Lineweaver-Burk plot. B. Hill plot. Experimental conditions were the same as described in METHODS, except that KCl was varied from 1 to 10 mM. Each point represents the mean of two separate experiments. ■——■, control; ●——●, .06 mM flupenthixol; ○——○, .09 mM fluphenazine.

TABLE 1

Kinetic parameters for potassium activation of K+-dependent p-nitrophenyl phosphatase

$K_{0.5}$, the substrate concentration at half-maximal velocity, is given by $K_{0.5} = K^{1/n}$, where K is the intercept and n is the slope of the Hill plot. n and K were calculated by the method of least squares. V_{max} was estimated graphically from a Lineweaver-Burk plot.

Addition	V_{max}	n	$K_{0.5}$
	μmoles P_i/mg protein/hr		*mM*
None	30.8	1.18	1.66
60 μM flupenthixol	30.8	1.36	9.19
90 μM fluphenazine	30.8	1.48	8.30

needed to produce this effect are higher than those reported to be effective when formation of the free radical was induced. However, in view of the cerebral level reached by substituted phenothiazines after systemic administration, a concentration of 0.1 mM can be estimated: a pharmacological level usually achieved in the studies *in vivo* (1).

The role of Mg·ATP in promoting ATPase inhibition and the antagonism by sodium and potassium give some indications of the molecular mechanism underlying the drug-induced effect. Kinetic analysis shows that inhibition by both flupenthixol and fluphenazine is uncompetitive with respect to Mg·ATP. Uncompetitive inhibition generally assumes exclusive binding of the inhibitor molecule to the enzyme-substrate complex (19). It is therefore conceivable that binding of Mg·ATP is followed by a conformational change which greatly facilitates interaction of these drugs with (Na+ + K+)-ATPase. A cooperative response of transport ATPase to Mg·ATP has already been considered on the basis of kinetic evidence (22) and would be in line with current views on the allosteric nature of the (Na+ + K+)-ATPase system. Anomalous kinetics with Mg·ATP was not found in more recent experiments (33). The competitive antagonism by sodium and potassium suggests that the drugs prevent the enzyme from assuming the conformation induced by the activating ions. As a consequence, Na+-dependent phosphorylation is decreased. An increased degree of cooperativity and enhanced $K_{0.5}$ for potassium activation of the phosphatase reaction were also con-

TABLE 2

Effects of flupenthixol and fluphenazine on sodium-dependent phosphorylation and $(Na^+ + K^+)$-ATPase activity

The reaction mixture for the labeling reaction contained, in a final volume of 0.5 ml, 200 mM imidazole HCl (pH 7.4), 0.5 mM $MgCl_2$, 100 mM NaCl, 0.5 mM [^{32}P]ATP-Tris (specific activity, 3×10^4 cpm/nmole), and 0.5 mg of enzyme preparation. The value of non-sodium-dependent phosphorylation (20–25% of the total phosphorylation), obtained by replacing 100 mM NaCl with 20 mM KCl, was subtracted. The $(Na^+ + K^+)$-ATPase reaction was carried out with 0.5 mM ATP, 0.5 mg of enzyme preparation, and 2 mM phosphoenolpyruvate plus 30 μg of pyruvate kinase to prevent substrate depletion. Other details were the same as described in METHODS. Under such conditions $(Na^+ + K^+)$-ATPase activity was 26 μmoles of P_i per milligram of protein per hour.

Drug tested	Concentration	$^{32}P_i$ incorporated	Inhibition	Inhibition of $(Na^+ + K^+)$-ATPase
	mM	*pmoles/mg protein*	*pmoles/mg protein (%)*	*%*
Flupenthixol	0	170		
	0.05	155	15 (9)	2.5
	0.1	152	18 (11)	5
	0.15	134	36 (26)	11
	0.25	122	48 (28)	12
Fluphenazine	0	166		
	0.1	150	16 (10)	2
	0.2	147	19 (11)	12
	0.35	128	38 (23)	14

TABLE 3

Effects of flupenthixol and fluphenazine on steady-state level of ^{32}P-labeled enzyme in the presence of 100 mM NaCl and 20 mM KCl

The procedure is described in the legend to Table 2. The value of nonspecific $^{32}P_i$ incorporation, determined in the presence of 20 mM KCl alone, was subtracted.

Addition	$^{32}P_i$ incorporation	
	Total	Decrease
	pmoles/mg protein	
None	185	
20 mM KCl	105	
20 mM KCl + 0.05 mM flupenthixol	89	16
20 mM KCl + 0.1 mM flupenthixol	84	21
20 mM KCl + 0.2 mM flupenthixol	63	42
None	170	
20 mM KCl	102	
20 mM KCl + 0.1 mM fluphenazine	82	20
20 mM KCl + 0.2 mM fluphenazine	69	33
20 mM KCl + 0.35 mM fluphenazine	63	39

sistently found. The antagonism by either sodium or potassium would suggest two distinct steps in the drug-induced inhibition. An alternative explanation is indicated by the "simultaneous" reaction mechanism for $(Na^+ + K^+)$-ATPase recently proposed by Whittam and Chipperfield (34). In such a "one-step" reaction mechanism, both sodium and potassium act at the same step preceding phosphorylation. Additional possibilities include the inhibition by flupenthixol and fluphenazine of phospholipid-induced activation of transport ATPase. In a study of oligomycin-induced inhibition of the phospholipid-dependent mitochondrial ATPase complex, it was shown that phospholipids can competitively remove the inhibitory effect (35) and that oligomycin sensitivity varies with exogenous phospholipid composition (36). It was proposed (37) that oligomycin can find in the lipid portion of the membrane an environment suitable for reaching the hydrophobic portion of the enzyme embedded in the lipid bilayer. Such a mechanism might be of general interest with respect to pharmacological effect induced by lipophilic drugs. $(Na^+ + K^+)$-

ATPase is a membrane-bound enzyme which requires phospholipids for activity. Recent studies on delipidated preparations have shown that activation by both sodium and potassium is phospholipid-dependent (38–40). The present experiments, as well as those of Roufogalis (8), show that the lipophilic forms of these drugs are more effective, and tricyclic antipsychotic agents are known to increase the fluidity of membrane phospholipids (41). In line with this possibility, it has been shown that changes in the fluidity of phospholipid acyl chains modify the allosteric properties of $(Na^+ + K^+)$-ATPase (42).

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