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PHOSPHOLIPID-SENSITIVE CALCIUM-DEPENDENT PROTEIN KINASE: INHIBITION BY ANTI-PSYCHOTIC DRUGS.

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SUMMARY: Phospholipid-sensitive Ca^{2+}-dependent protein kinases partially purified from the rat cerebral cortex, pig spleen, and bovine heart were shown to be inhibited, to varying degrees, by several antipsychotic drugs including trifluoperazine, chlorpromazine, fluphenazine, haloperidol, and chlorprothixene and by the local anesthetic dibucaine. None of these drugs were found to have any significant effect on cyclic AMP-dependent and cyclic GMP-dependent protein kinases. Kinetic analysis suggests that the primary effect of the drugs is mediated through a competitive inhibition of enzyme activation by interacting with phosphlipid.

Calcium ion exerts a profound influence on a wide variety of biological processes (for reviews, see Ref. 1 and 2). However, the exact mechanism by which calcium exerts its effects remains obscure. Evidence acquired in several laboratories over the last few years suggests that calmodulin, a multifunctional Ca^{2+}-binding protein, is an important mediator of the actions of calcium (for reviews, see Ref. 3 and 4). Levin and Weiss (5) have demonstrated that certain antipsychotic drugs can bind to the Ca^{2+}-calmodulin complex and, thereby, inhibit the complex from interacting with and activating its target enzymes. The suggestion has been made (5,6) that the interactions of these drugs with calmodulin systems may explain some of their antipsychotic and side effects and, additionally, that these agents may serve as important tools with which to investigate the actions of calcium in biological systems.

Recently, Takai et al. (7) have reported a protein kinase which is activated by Ca^{2+} in the presence of phospholipid (such as phosphatidylserine). Evidence from our laboratory (8,9) has shown a widespread occurrence of this phospholipid-

[1]The abbreviations used are: Ca-PK, Ca^{2+}-dependent protein kinase; A-PK, cyclic AMP-dependent protein kinase; G-PK, cyclic GMP-dependent protein kinase; A-PDE, cyclic AMP phosphodiesterase.

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sensitive Ca-PK[1] (8) as well as its endogenous substrates (9) in various tissues and phyla of the animal kingdom, and suggests that calmodulin apparently is not involved in the action of Ca^{2+} on this system. Mori et al. (10) recently demonstrated that drugs shown to interact with phospholipids (11), such as chlorpromazine and dibucaine, could inhibit the activity of this enzyme system, presumably by interacting with phospholipid. The present study was conducted to investigate which antipsychotic drugs, in addition to chlorpromazine, could inhibit this enzyme system and to compare the effects of these drugs on phospholipid-sensitive and calmodulin-sensitive enzyme reactions.

EXPERIMENTAL PROCEDURE

Materials: Phosphatidylserine (bovine brain), lysine-rich histone (type III-S), mixed histone (type II), 1,3-diolein, cAMP, cGMP, chlorpromazine·HCl, and dibucaine·HCL were obtained from Sigma; cyclic [G-^{3}H]AMP was from New England Nuclear. Trifluoperazine·2HCl was obtained through Dr. P. T. Ridley of Smith, Kline and French; fluphenazine·2HCl was from the Squibb Institute for Medical Research; haloperidol was from McNeil Laboratories; chlorprothixene was the gift of Dr. Benjamin Weiss of the Medical College of Pennsylvania.

Purification of Phospholipid-sensitive Ca-PK: Rat cerebral cortex, pig spleen, and bovine heart were each homogenized in 3 volumes of ice-cold 20 mM Tris/Cl, pH 7.5, containing 2 mM EDTA and 50 mM 2-mercaptoethanol. The homogenates were centrifuged for 20 min. at 30,000 x g and the supernatant fluids (extracts) were used in the subsequent purification steps. Phospholipid-sensitive Ca-PK was purified about 100-fold from rat cerebral cortex extract by Sephadex G-200 and DEAE-cellulose chromatographies, about 200-fold from pig spleen extract by DEAE-cellulose and octyl-agarose chromatographies, and about 500-fold from bovine heart extract by ammonium sulfate precipitation followed by DEAE-cellulose, controlled-pore glass, and Sephadex G-200 chromatographies (unpublished).

Phospholipid-sensitive Ca^{2+}-PK was assayed as previously described (8). Briefly, the assay system, in a final volume of 0.2 ml, contained Tris/Cl, pH. 7.5, 5 µmol; lysine-rich histone, 40 µg; $MgCl_2$, 2 µmol; phosphatidylserine, 1.5 µg; 1,3-diolein, 0.15 µg; $CaCl_2$, 0.04 µmol; 5-20 µg phospholipid-sensitive Ca-PK from rat cerebral cortex, pig spleen, or bovine heart purified as described above. The reaction was initiated by addition of [γ-^{32}P]ATP, 1 nmol, containing 0.5-1.0 x 10^6 cpm. The reaction was carried out for 5 min at 30°.

Other Methods: A-PK was purified from bovine heart through the hydroxylapatite step as previously described (12). G-PK was purified through the DEAE-cellulose step as previously described (13). A-PK and G-PK were assayed as described previously (14). Calmodulin-sensitive A-PDE was prepared by DEAE-cellulose chromatography as described earlier (9). A-PDE was assayed essentially as described previously (15), using 6 units of pure calmodulin (1 unit being defined as that amount of protein giving half-maximal stimulation of calmodulin-sensitive Ca^{2+}-dependent A-PDE). The PDE activity values were corrected for the blank value seen in the absence of added enzyme.

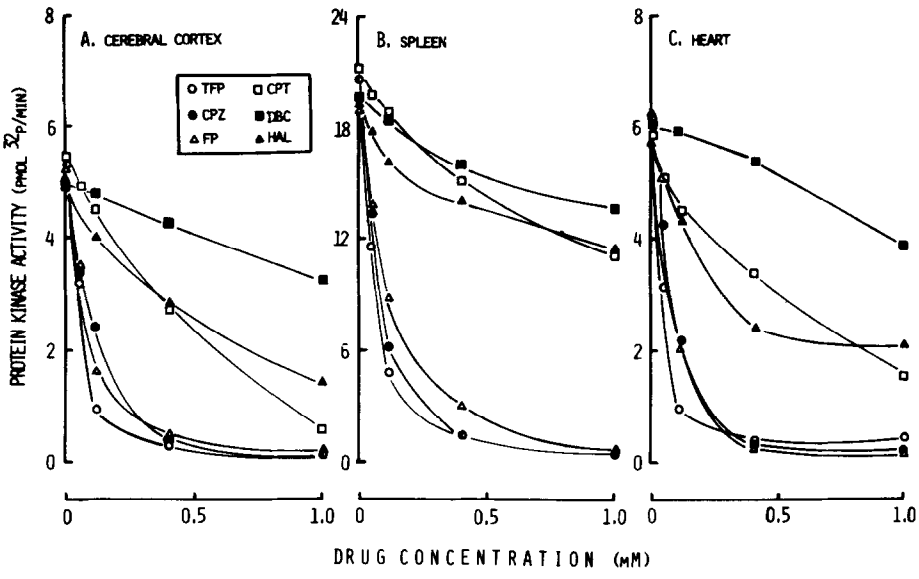

Fig. 1. Inhibition of phospholipid-sensitive Ca-PK by various drugs. Conditions were as described under Experimental Procedure, with various concentrations of drugs added as indicated. The protein kinases employed were partially purified from rat cerebral cortex, pig spleen, and bovine heart; the amounts of protein used were 6, 15, and 7 μg, respectively. TFP, trifluoperazine; CPZ, chlorpromazine; FP, fluphenazine; CPT, chlorprothixene; DBC, dibucaine; HAL, haloperidol.

All drugs used in the current study were solubilized in water, except for chlorprothixene and haloperidol which were dissolved in 95% ethanol and fluphenazine which was dissolved in 25% ethanol. The addition of an equivalent amount of ethanol to the phospholipid-sensitive Ca-PK reaction mixture increased the basal (no Ca^{2+} added) enzyme activity by 75%, however, it had no effect on the total stimulated (Ca^{2+} added) enzyme activity. Additionally, the presence of ethanol in the reaction mixture did not prevent either the activation of the enzyme by Ca^{2+} or the inhibition of the activation by the drugs. The IC_{50} was defined as that drug concentration which produced 50% inhibition of the maximally stimulated enzyme activity.

Homogeneous calmodulin was prepared from bovine brain by the fluphenazine affinity method described by Charbonneau and Cormier (16). [γ-^{32}P]ATP was prepared by the method of Post and Sen (17). Protein was determined by the method of Bradford (18), using ovalbumin as a standard protein.

RESULTS AND DISCUSSION

The activity of phospholipid-sensitive Ca-PK partially purified from rat cerebral cortex, pig spleen, and bovine heart was inhibited, to varying degrees, by several antipsychotic drugs and dibucaine (Fig. 1). Trifluoperazine appeared to be the most potent inhibitor, with an IC_{50} of 38-50 μM under the assay conditions employed (Table 1). Chlorpromazine and fluphenazine were less potent, with IC_{50}

TABLE I

Summary of IC_{50} values for the drugs of phospholipid-sensitive Ca-PK and calmodulin-sensitive A-PDE

The values of Ca-PK were calculated from experiments presented in, or similar to, Fig. 1., using drug concentrations up to 10 mM.

Drugs	IC_{50} (μM)						
	Phospholipid-sensitive Ca-PK						Calmodulin-sensitive A-PDE
	Cerebral cortex		Spleen		Heart		
	-Ca	+Ca	-Ca	+Ca	-Ca	+Ca	
Trifluoperazine	33	45	32	50	24	38	10[a],12[b]
Chlorpromazine	39	84	81	59	41	65	42[a]
Fluphenazine	36	58	90	114	30	78	19[b]
Chlorprothixene	100	335	288	>1000[c]	273	600	16[a]
Haloperidol	380	510	400	>1000[c]	376	362	60[d]
Dibucaine	540	>1000	760	>1000	687	1000	313[b]

[a]Taken from Levin and Weiss (5); [b]Determined in the present studies, see the text; [c]Drugs not soluble above 2 mM; [d]Taken from Weiss and Levin (6).

values of 59-84 μM and 58-114 μM, respectively. Chlorprothixene, haloperidol, and dibucaine were much less potent, with IC_{50} values 6 to 30 times greater than those for trifluoperazine. In each case, the IC_{50} values for the various drugs inhibiting the phospholipid-sensitive Ca-PK were higher, in some instances many fold higher, than their respective IC_{50} values for the inhibition of the calmodulin sensitive Ca^{2+}-dependent A-PDE (Table I). Additionally, the data in Table I demonstrate that there is no correlation between the relative order of potency of the antipsychotic drugs for the inhibition of phospholipid-sensitive Ca-PK and for the inhibition of A-PDE as reported earlier by Levin and Weiss (5). These findings appear to support the contention that calmodulin is not involved in the phospholipid-sensitive Ca-PK reaction, and that the drugs interact somewhat differently with phospholipid and calmodulin

Studies were conducted to determine if these drugs would also inhibit other classes of protein kinases. The data presented in Table II clearly indicate that trifluoperazine, while nearly completely inhibiting phospholipid-sensitive Ca-PK, had little or no effect on A-PK and G-PK.

Next, experiments were carried out to further investigate the mechanism by which these drugs exert their inhibitory effect. As shown in Fig 2A, high

TABLE II

Effects of trifluoperazine on various protein kinases

The enzymes were assayed in the presence or absence of $CaCl_2$ (0.5 mM), cGMP (0.5 μM) and cAMP (0.5 μM), as indicated. The enzymes were all from the bovine heart: phospholipid-sensitive Ca-PK, 7 μg; G-PK, 10 μg; A-PK, 10 μg.

Trifluoperazine (μM)	Protein kinase activity (pmol ^{32}P/min)					
	Ca-PK		G-PK		A-PK	
	-Ca	+Ca	-cGMP	+cGMP	-cAMP	+cAMP
0	2.1	10.7	2.5	10.7	1.8	17.2
40	0.9	5.5	2.4	10.3	2.4	16.2
80	0.7	2.3	2.8	9.0	2.7	16.3

concentrations of phosphatidylserine were able to totally overcome the inhibition of Ca-PK caused by 40 μM of trifluoperazine. This was accompanied by an increased K_a for phosphatidylserine from 7 μg/ml in the absence of the drug to 17 μg/ml in its presence. These data suggest that the inhibitory action of trifluoperazine was competitive with phosphatidylserine. However, as shown in Fig 2B, increasing con centrations of $CaCl_2$ were unable to totally overcome the effect of trifluoperazine.

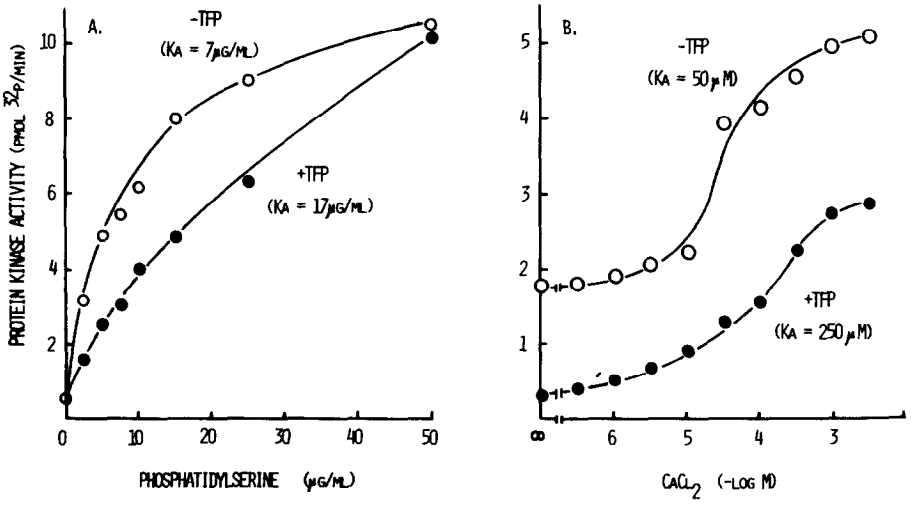

Fig. 2. Effect of trifluoperazine (TFP) on phospholipid-sensitive Ca-PK as a function of phosphatidylserine or $CaCl_2$ concentrations. The enzyme (6 μg), from the rat brain, was assayed as described in Experimental Procedure, except for the varying concentrations of phosphatidylserine and $CaCl_2$ and inclusion of trifluoperazine (60 μM). A, contained 50 μM $CaCl_2$; B, contained 1.5 μg phosphatidylserine and 0.15 μg diolein.

TABLE III

Effects of phosphatidylserine on inhibition by trifluoperazine of calmodulin-sensitive A-PDE, and effect of calmodulin on inhibition by trifluoperazine of phospholipid-sensitive Ca-PK.

In Experiment I, A-PDE (10 μg), from the rat brain, was activated by 6 units of calmodulin and 50 μM of $CaCl_2$. In Experiment II, Ca-PK (6 μg), from the rat brain, was activated by 1.5 μg of phosphatidylserine, 0.15 μg of diolein and 50 μM of $CaCl_2$.

Addition	Trifluoperazine (μM)		
	0	20	40
Experiment I: A-PDE (pmol cAMP/min)			
None	1.8	0.7	
Phosphatidylserine, 1.5 μg	1.8	1.0	
Phosphatidylserine, 15 μg	1.9	1.8	
Experiment II: Ca-PK (pmol ^{32}P/min)			
None	6.5		3.5
Calmodulin, 120 units	6.5		3.6

In addition, the K_a of the enzyme for Ca^{2+} was increased 5-fold (from 50 to 250 μM) in the presence of the drug. These data are difficult to interpret in view of the complexity of the reaction system, therefore, at this time the exact mechanism of action of trifluoperazine on the involvement of Ca^{2+} with the enzyme cannot be stated. The data shown in Fig. 2 are in line with that reported earlier by Mori et al. (10) for chlorpromazine and other lipid-interacting drugs.

Further evidence for trifluoperazine interacting with phospholipid is shown in Table III, Experiment I. Trifluoperazine (20 μM) inhibited A-PDE by 60%. Phosphatidylserine (50 μg), while having no effect on control A-PDE activity seen in the absence of the drug, was able to totally overcome the inhibition caused by the drug. A lower amount of phosphatidylserine (1.5 μg) was able to partially overcome the inhibition.

Experiments were also conducted to test whether calmodulin, previously shown to bind trifluoperazine to a high degree (5), would overcome the inhibition of Ca-PK by trifluoperazine. As shown in Table III, Experiment II, 40 μM of the drug caused a 45% inhibition of the Ca-PK activity. Calmodulin (120 units, which was 20 times higher than the amount causing maximal stimulation of A-PDE), while

having no appreciable effect on control Ca-PK activity seen in the absence of the drug, was unable to overcome the inhibition of Ca-PK caused by the drug. The reason for this result remains unclear, though the possibility exists that higher concentrations of calmodulin might overcome the inhibition.

The present studies indicate that several antipsychotic drugs were able not only to inhibit Ca^{2+}-calmodulin requiring processes, as shown by others (5,6,19) and by us (Table I), but also to inhibit a phospholipid-sensitive Ca-PK partially purified from various tissues. Levin and Weiss (5) have previously shown that certain antipsychotic drugs appear to bind to the Ca^{2+}-calmodulin complex and make it a poor activator of calmodulin-sensitive A-PDE. Our data and that of Mori et al. (10) suggest that the primary effect of these drugs to inhibit the phospholipid-sensitive Ca-PK is through a competitive interaction with the phospholipid cofactor. Since the relative potency of the drugs for the inhibition of the Ca-PK and A-PDE activities was not the same (Table I), it is further suggested that the drugs may interact with the two cofactors (i.e. phospholipid and calmodulin) in dissimilar manners.

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