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Mechanism by Which Psychotropic Drugs Inhibit Adenosine Cyclic 3',5'-Monophosphate Phosphodiesterase of Brain

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SUMMARY

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Bovine brain contains several molecular forms of adenosine cyclic 3',5'-monophosphate phosphodiesterase. The activity of one of these forms is increased several fold by an endogenous protein activator. Chlorpromazine, a potent phenothiazine tranquilizer, specifically inhibits the activation of a partially purified phosphodiesterase prepared from bovine brain at concentrations which do not inhibit the enzyme in the absence of activator. Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA), a calcium chelator, also specifically inhibits the activation of phosphodiesterase. However, the mechanisms by which EGTA and chlorpromazine act are different. EGTA produces its effect by chelating calcium, a required cofactor for phosphodiesterase activation; the EGTA-induced inhibition can be overcome by increasing the calcium concentration but not by increasing the concentration of activator. In contrast, chlorpromazine inhibits the activation of phosphodiesterase by interfering with the phosphodiesterase-activator interaction; the chlorpromazine-induced inhibition can be overcome by increasing the concentration of activator but not by increasing the concentration of calcium. Several other psychotropic drugs also inhibit the activation of phosphodiesterase of bovine brain. The antipsychotic agents trifluoperazine, thioridazine, benperidol, pimozide, and chlorprothixene are all potent, selective inhibitors of the activation of phosphodiesterase. The antianxiety agents medazepam and chlordiazepoxide and the antidepressants amitriptyline, protriptyline, and desipramine also selectively inhibit the activation of phosphodiesterase but are significantly less potent than the antipsychotics. Phenothiazines with relatively weak antipsychotic actions, chlorpromazine sulfoxide and promethazine, are less selective and less potent inhibitors than the antipsychotic phenothiazine derivatives. Other agents having central pharmacological actions, such as pentobarbital, pipradrol, D-lysergic acid diethylamide, pentylenetetrazol, morphine, and amphetamine, are poor inhibitors of either the activated or unactivated phoshodiesterase. These findings suggest that (a) the mechanism by which the phenothiazines inhibit the activation of phosphodiesterase involves competition with the endogenous protein activator of phosphodiesterase, and (b) selective inhibition of the activation of phosphodiesterase is a property common to agents which are effective in certain forms of psychiatric illnesses rather than to either general central nervous system depressants or stimulants.

INTRODUCTION

Mammalian brain contains several molecular forms of adenosine cyclic 3',5'-monophosphate phosphodiesterase (1-11). These forms of phosphodiesterase have different patterns of activity in different

brain regions (5–7), develop at different rates ontogenetically (9), and have a number of distinguishing physical and chemical characteristics, including different molecular weights (1–3), stabilities (1, 5, 6), and substrate specificities (1–4). These forms of phosphodiesterase can also be differentially inhibited by pharmacological agents (5–7, 12) and differentially activated by an endogenous protein activator (5–14). The activation process, which is dependent upon the presence of calcium (15–18), is particularly interesting since it appears to be fairly selective for only one of the several forms of phosphodiesterase found in brain (5–7, 10).

Recently it was found that the activation of phosphodiesterase can be selectively inhibited by two distinct types of inhibitors. One type is represented by the calcium chelator, EGTA.[1] EGTA prevents the calcium-dependent activation of phosphodiesterase and inhibits the enzymatic activity of phosphodiesterase if it already is in the activated state (16–18). Another type of compound which inhibits the activation of phosphodiesterase is exemplified by the phenothiazine tranquilizer trifluoperazine (5–7). This compound selectively inhibits the activation of phosphodiesterase at concentrations which have little effect on the enzymatic activity in the absence of activator (7, 19).

Thus two compounds, EGTA and trifluoperazine, share the property of inhibiting the activation of phosphodiesterase. The studies reported here seek to clarify the following two points: (a) Do EGTA and the phenothiazines act by the same mechanism? (b) Do psychotropic agents other than trifluoperazine share this property of selectively inhibiting the activation of phosphodiesterase?

METHODS

Phosphodiesterase activity. Two sources of phosphodiesterase were used in these studies: a partially purified, soluble, activator-deficient phosphodiesterase prepared from bovine brain (obtained fresh from a local slaughterhouse) according to Teo, Wang, and Wang (14), and a highly purified phosphodiesterase separated from rat brain (Sprague-Dawley) by polyacrylamide gel electrophoresis (5). In all instances the results obtained using either phosphodiesterase preparation were similar.

Phosphodiesterase activity was measured by the luciferin-luciferase method as previously described (20). Each reaction vessel contained 50 mM glycylglycine buffer (pH 8.0), 25 mM ammonium acetate, 3 mM $MgCl_2$, 1.8 units of myokinase, 0.2 unit of pyruvate kinase, 400 μM cAMP, 100 μM $CaCl_2$, and the phosphodiesterase preparation in a total volume of 160 μl. The 5′-AMP standards were incubated under the same conditions as the phosphodiesterase so that corrections could be made for any influence the compounds under study might have on the assay system.

Activator activity. Activator was purified from bovine brain according to Teo *et al.* (14). One unit of activator was defined as the amount necessary to produce 50% of the maximum activation of the activator-deficient phosphodiesterase attainable under standard experimental conditions (described above). This proved to be a consistent and reliable means of quantitating the activity of the protein activator.

Pharmacological agents. Before the reaction was started, 10 μl of pharmacological agents were added to the incubation medium. Benperidol and pimozide were dissolved in 95% ethanol. The addition of an equivalent amount (10 μl) of ethanol to the reaction mixture inhibited the enzymatic activity by approximately 40% but did not prevent either the activation of phosphodiesterase by activator or the inhibition of activation by the pharmacological agents. All other drugs were used in aqueous solutions.

The concentration of a drug which produced 50% inhibition of phosphodiesterase activation in the presence of 10 units of activator is defined as I_{50} (activated) for that drug. I_{50} (unactivated) is defined as the concentration of drug which produced 50% inhibition of phosphodiesterase in the absence of activator.

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate.

Protein concentrations were measured by the method of Lowry *et al.* (21).

Materials. Pyruvate kinase, myokinase, and phosphoenolpyruvate were obtained from Boehringer/Mannheim Corporation, and firefly luciferin and luciferase, from du Pont de Nemours and Company. Other reagents were obtained from general commercial sources. Chlorpromazine, chlorpromazine sulfoxide, and trifluoperazine were kindly supplied by Smith Kline & French Laboratories; medazepam, chlordiazepoxide, and chlorprothixene, by Hoffmann-La Roche, Inc.; pimozide, by McNeil Laboratories, Inc.; thioridazine, by Sandoz Pharmaceuticals; promethazine, by Wyeth Laboratories; amitriptyline and protriptyline, by Merck Sharp & Dohme; desipramine, pipradrol, and azacyclonol, by Merrell National Laboratories; and benperidol, by Janssen Pharmaceuticals.

RESULTS

Inhibition of phosphodiesterase activity by chlorpromazine in the presence and absence of activator. Figure 1 demonstrates the selective inhibition of the activation of phosphodiesterase by chlorpromazine. The addition of 10 units of activator to the phosphodiesterase preparation increased the enzymatic activity approximately 4-fold. Increasing the concentration of chlorpromazine progressively inhibited the activation of phosphodiesterase. The concentration of chlorpromazine which inhibited the activation of phosphodiesterase by 50% [I_{50} (activated)] was 42 μM. In the concentration range examined, chlorpromazine failed to inhibit the activity of phosphodiesterase in the absence of activator (control sample).

Effect of calcium on chlorpromazine-induced inhibition of phosphodiesterase activation. Since the activation of phosphodiesterase requires calcium, and since chlorpromazine can complex calcium (22), we determined whether increasing the concentration of calcium could prevent the chlorpromazine-induced inhibition of phosphodiesterase activation. For comparative purposes we also measured the effect of calcium on the EGTA-induced inhibition of phosphodiesterase activation (Fig. 2).

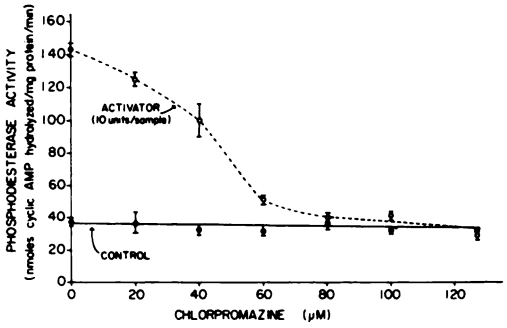

FIG. 1. *Inhibition of phosphodiesterase activity by chlorpromazine in the presence and absence of activator*

Phosphodiesterase activity of a preparation partially purified from bovine brain was measured in the absence of activator (control) and in the presence of 10 units of activator and varying concentrations of chlorpromazine. Each point is the mean of six determinations. Vertical brackets represent standard errors.

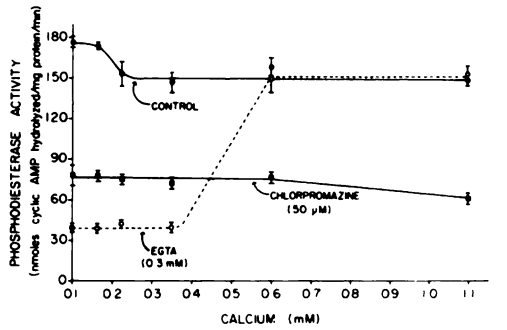

FIG. 2. *Effect of increasing calcium concentration on inhibition of activation of phosphodiesterase induced by chlorpromazine or EGTA*

Phosphodiesterase activity of a preparation partially purified from bovine brain was measured in the absence of any inhibitors (control) and in the presence of 50 μM chlorpromazine or 0.3 mM EGTA and varying concentrations of calcium. Ten units of the phosphodiesterase activator were present in all samples. Each point is the mean of three determinations. Vertical brackets represent standard errors.

In the presence of 0.1 mM calcium and 10 units of activator, 50 μM chlorpromazine inhibited the activity of phosphodiesterase by 60%. In a similar preparation, i.e., in the presence of 0.1 mM calcium and 10 units of activator, the addition of 0.3 mM EGTA inhibited the activity by nearly 80%; this phosphodiesterase activity obtained in the presence of EGTA and activator (i.e., 40 nmoles of cAMP hydrolyzed

per milligram of protein per minute) was the same as that measured in the absence of activator (results not shown), suggesting that 0.3 mM EGTA totally inhibited the activation of phosphodiesterase. Increasing the concentration of calcium completely overcame the EGTA-induced inhibition of the activation of phosphodiesterase, but failed to prevent the chlorpromazine-induced inhibition of phosphodiesterase activation (Fig. 2). In the control samples, i.e., when phosphodiesterase was measured in the absence of chlorpromazine or EGTA, calcium produced a slight (about 10%) inhibition of phosphodiesterase activity.

Effect of increasing activator concentration on chlorpromazine-induced inhibition of phosphodiesterase activation. We next determined whether the chlorpromazine-induced inhibition of phosphodiesterase activation could be overcome by increasing the concentration of activator. Figure 3 shows the effect of activator on phosphodiesterase activity in the presence and absence of 50 μM chlorpromazine or 0.3 mM EGTA. In the absence of inhibitors (control sample), increasing the concentration of activator produced a concentration-dependent increase in phosphodiesterase activity, which reached a maximum elevation of about 6-fold. In the absence of added activator, 0.3 mM EGTA produced about 40% inhibition of phosphodiesterase activity, suggesting that a portion of this enzyme preparation was partially activated. Increasing the concentration of activator in the presence of EGTA failed to increase the activity of phosphodiesterase.

In the absence of activator, 50 μM chlorpromazine inhibited phosphodiesterase activity to the same extent as did EGTA. Increasing the concentration of activator in the presence of chlorpromazine progressively increased phosphodiesterase activity. At an activator concentration of 1 unit/sample, chlorpromazine inhibited activation by 62.8%, while at 40 units of activator per sample, 50 μM chlorpromazine inhibited the activation of phosphodiesterase by only 17%. These results suggest that the activator may competitively antagonize the chlorpromazine-induced inhibition of the activation of phosphodiesterase.

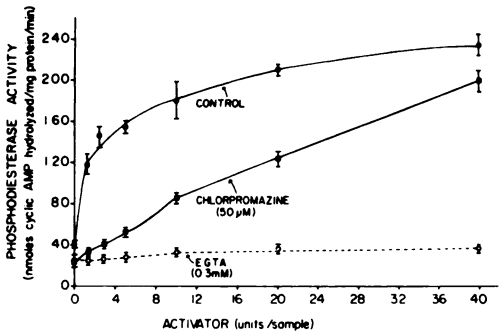

FIG. 3. *Effect of increasing activator concentration on inhibition of activation of phosphodiesterase induced by chlorpromazine or EGTA*

Phosphodiesterase activity of a preparation partially purified from bovine brain was measured in the absence of any inhibitors (control) and in the presence of 50 μM chlorpromazine or 0.3 mM EGTA and varying concentrations of activator. Each point is the mean of three determinations. Vertical brackets represent standard errors.

To investigate further this interaction among activator, phosphodiesterase, and chlorpromazine, the activity of phosphodiesterase was determined at various concentrations of activator and chlorpromazine. The data (Fig. 4) were plotted as $1/v$ vs. 1/activator concentration, and the lines of best fit were determined by linear regression analysis. The point at which the lines cross the y axis represents the theoretical maximum velocity (V_{max}) obtained in the presence of activator. As can be seen, the V_{max} values for phosphodiesterase in the absence and presence of 20 or 60 μM chlorpromazine were similar. On the other hand, the apparent affinity of the activator for the phosphodiesterase decreased with increasing concentrations of chlorpromazine. This is consistent with the idea that chlorpromazine is a competitive inhibitor of phosphodiesterase activation.

Similar results were obtained in studies of the effects of trifluoperazine on a highly activatable form of phosphodiesterase purified from rat cerebrum by polyacrylamide gel electrophoresis (Fig. 5). Increasing the concentration of activator overcame the inhibition of phosphodiesterase activity induced by trifluoperazine. As with chlorpromazine, the calculated V_{max} was the same for varying concentrations of trifluopera-

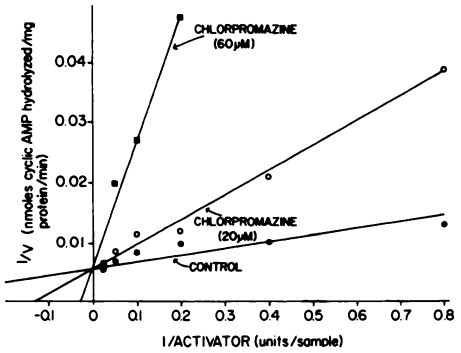

FIG. 4. *Kinetic analysis of chlorpromazine-induced inhibition of activation of phosphodiesterase*

Phosphodiesterase activity of a preparation partially purified from bovine brain was measured in the presence of varying concentrations of activator and chlorpromazine. Velocities shown are the increase in phosphodiesterase activity caused by the activator. Each point is the mean of six determinations.

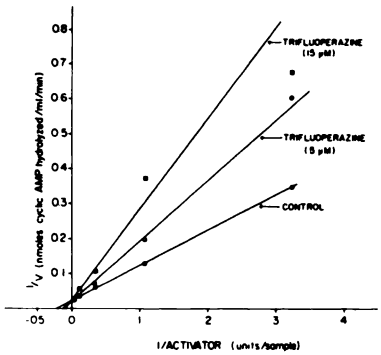

FIG. 5. *Kinetic analysis of trifluoperazine-induced inhibition of activation of phosphodiesterase*

The activity of an activatable phosphodiesterase (peak II) isolated from rat cerebrum by polyacrylamide gel electrophoresis was measured in the presence of varying concentrations of activator and trifluoperazine. Each point is the mean of four determinations.

zine, suggesting competition with the activator.

Comparison of effects of chlorpromazine on cAMP and cGMP phosphodiesterase activities. The enzyme preparation we used in these experiments hydrolyzes cGMP as well as cAMP. Since it has been shown (23, 24) that the protein activator also activates cGMP phosphodiesterase, we compared the effects of chlorpromazine on blocking the activation of cAMP phosphodiesterase (400 μM cAMP as substrate) and cGMP phosphodiesterase (200 μM cGMP as substrate). Our results showed that the phenothiazine was about 3 times more effective in blocking the activation of cAMP phosphodiesterase (I_{50} = 45 μM) than the activation of cGMP phosphodiesterase (I_{50} = 140 μM).

Effects of various psychotropic agents on phosphodiesterase activity in the presence and absence of activator. The specificity and potency of the phenothiazines in inhibiting the activated form of phosphodiesterase led us to inquire whether other psychoactive agents might share this property. Several psychotropic agents having diverse structures and representing several different classes of compounds were examined for their ability to inhibit phosphodiesterase in the presence and absence of activator. The I_{50} values for these agents were determined as described in METHODS. Determination of some I_{50} values was limited by the solubility of the agents.

Of the compounds studied, only the antipsychotic agents had I_{50} values under 100 μM (Table 1). The most potent was pimozide (7 μM), and the least potent was benperidol (58 μM); trifluoperazine, chlorpromazine, thioridazine, and chlorprothixene were intermediate in potency. In the concentrations studied, these agents inhibited the unactivated form of phosphodiesterase only slightly. The antianxiety agents chlordiazepoxide and medazepam and the antidepressants amitriptyline, protriptyline, and desipramine were also selective inhibitors of the activated form of phosphodiesterase. However, they were considerably less potent than the antipsychotics, with I_{50} values ranging between 125 and 320 μM.

Chlorpromazine sulfoxide, a phenothiazine with weak antipsychotic effects, was a far less potent and specific inhibitor of the activation of phosphodiesterase than its more potent counterpart. Promethazine, a phenothiazine with antihistaminic and sedative properties, had inhibitory characteristics similar to those of the antianxiety agents. The widely studied phosphodiesterase inhibitors theophylline and papaverine showed little preference for either form of phosphodiesterase.

In the concentrations studied, other cen-

TABLE 1

Effects of several pharmacological agents on activated and unactivated phosphodiesterase of bovine brain

Phosphodiesterase activity of a preparation partially purified from bovine brain was measured in the presence and absence of 10 units of activator and various concentrations of the compounds under study, using 400 μM cAMP as substrate. In these studies the phosphodiesterase preparation of each sample contained 50 ng of protein. At this enzyme concentration, EGTA did not reduce the basal phosphodiesterase activity, suggesting that the enzyme was in an unactivated state. The addition of 10 units of activator produced about a 4-fold increase in phosphodiesterase activity. The I_{50} (activated) and I_{50} (unactivated) were calculated as described in METHODS. A minimum of six replicate samples at each of six concentrations of drug was used in each experiment. In some cases the maximum concentration used was limited by the solubility of the compound.

Class	Drug	I_{50} (activated)	I_{50} (unactivated)
		μM	μM
Phenothiazines	Trifluoperazine	10	>300
	Thioridazine	18	>1000
	Chlorpromazine	42	>500
	Chlorpromazine sulfoxide	2500	7500
	Promethazine	340	>1000
Thioxanthene	Chlorprothixene	16	>2000
Butyrophenone	Benperidol	58	>300
Diphenylbutylamine	Pimozide	7	>100
Benzodiazepines	Medazepam	150	>1000
	Chlordiazepoxide	320	2500
Dibenzazepines	Amitriptyline	130	>1000
	Protriptyline	200	>1000
	Desipramine	125	>1000
Classical phosphodiesterase inhibitors	Theophylline	1500	2500
	Papaverine	130	210
Other centrally active agents	Amphetamine	>10 mM	>10 mM
	Mescaline	>5 mM	>5 mM
	Pentylenetetrazol	>10 mM	>10 mM
	D-Lysergic acid diethylamide	>2.5mM	>2.5mM
	Pipradrol	>1 mM	>1 mM
	Azacyclonol	>1 mM	>1 mM
	Pentobarbital	>10 mM	>10 mM
	Morphine	>4 mM	>4 mM

trally active compounds, such as amphetamine, D-lysergic acid diethylamide, mescaline, pentylenetetrazol, pipradrol, pentobarbital, azacyclonol, and morphine, were poor inhibitors of phosphodiesterase in both the absence and presence of activator.

DISCUSSION

The mechanism by which phenothiazine tranquilizers and other psychotropic agents act has been the subject of numerous investigations (11, 25–38). Recent studies suggest that they may produce their effects by altering the cyclic nucleotide system of brain (11, 26–38). Two opposing effects on the cyclic nucleotide system have been noted. On the one hand, these drugs can specifically block the elevation of cAMP in specific brain areas induced by decapitation (27, 30, 32), norepinephrine (26, 28–30, 32), or dopamine (31, 33, 35, 38). On the other hand, it was shown recently that phenothiazine tranquilizers not only block the elevation of cAMP, presumably by inhibiting adenylate cyclase stimulation (30, 39), but also inhibit the cyclic nucleotide phosphodiesterase system of brain, acting specifically on a form of the enzyme which is sensitive to an endogenous protein activator (5–7).

The present results confirm these initial findings on the phosphodiesterase system and show further that the mechanism by which the phenothiazines inhibit the activation of phosphodiesterase is distinct

from that produced by EGTA. The EGTA-induced inhibition of phosphodiesterase activation could be overcome by increasing the concentration of calcium but not by increasing the concentration of activator. In contrast, the chlorpromazine-induced inhibition was unaffected by increasing the calcium concentration but could be antagonized by increasing the concentration of activator. Kinetic studies of the interaction between activator and chlorpromazine (or trifluoperazine) showed that the V_{max} values of activated phosphodiesterase measured in the absence and presence of several concentrations of the phenothiazine were similar. The conclusions reached from these studies are that (a) EGTA inhibits the activation of phosphodiesterase by chelating calcium, a required cofactor for the activation process, and (b) chlorpromazine inhibits the activation of phosphodiesterase by interfering with the interaction of activator with phosphodiesterase in a competitive manner. Whether chlorpromazine binds to the activator to produce an inactive activator-chlorpromazine complex, or, alternatively, whether chlorpromazine binds to phosphodiesterase to prevent activator binding, is currently under investigation. Moreover, it should be noted that in these studies we examined only the soluble phosphodiesterase. Whether chlorpromazine has similar effects on the particulate phosphodiesterase has yet to be determined.

The data showing that chlorpromazine is more effective in blocking the activation of cAMP phosphodiesterase than that of cGMP phosphodiesterase support the notion, originally suggested by the data of Kakiuchi *et al.* (15) and Brostrom and Wolff (23, 24), that the activator has a higher binding affinity for the cGMP phosphodiesterase than for the cAMP phosphodiesterase. These experiments may provide the basis for selective inhibition of the cAMP and cGMP phosphodiesterases.

The effect of chlorpromazine on the activation of phosphodiesterase is shared by several other centrally active compounds of diverse chemical structure. The most potent of the selective inhibitors of the activation of phosphodiesterase were the antipsychotics. These included the phenothiazines trifluoperazine, thioridazine, and chlorpromazine; the butyrophenone benperidol; the thioxanthene chlorprothixene; and the diphenylbutylamine pimozide. Pharmacological agents classified as antianxiety agents and antidepressants were also selective inhibitors of the activator-sensitive phosphodiesterase, although they were less potent than the antipsychotics. A variety of other compounds having central pharmacological actions, including sedative-hypnotics and central nervous system stimulants, failed to exhibit any specificity for inhibiting the activation of phosphodiesterase. These limited studies suggest that the property of selectively inhibiting the activation of phosphodiesterase may be more common to drugs that are effective in certain forms of psychiatric diseases than to agents which act either as general central nervous system depressants or stimulants. However, whether these observations will have any relevance ultimately in explaining these pharmacological actions remains to be determined.

Thus phenothiazine antipsychotics and perhaps other psychotropic drugs have two major actions on the cAMP system of brain. They inhibit a specific, catecholamine-sensitive adenylate cyclase system and a specific, activator-sensitive phosphodiesterase system. A common factor in both the adenylate cyclase and phosphodiesterase systems may be the endogenous protein activator; Brostrom *et al.* (40) and Cheung *et al.* (41) have isolated a heat-stable activator of adenylate cyclase from brain which appears to be identical with the activator of phosphodiesterase. If the same protein activator is an integral component of a specific form of adenylate cyclase and a specific form of phosphodiesterase, it would seem reasonable that a single agent could affect both systems.

The findings that antipsychotic agents can inhibit the activity of both adenylate cyclase and phosphodiesterase may provide an explanation for some of the diverse pharmacological actions of these drugs. The net effect of these agents on the concentration of cAMP in specific areas of brain would be determined by the regional

distribution of both the hormone-sensitive adenylate cyclase and the activator-sensitive phosphodiesterase, and by the ratio of these two specific enzymes. In fact, it has already been shown that there are specific regions in brain which contain norepinephrine-sensitive adenylate cyclases (26, 27, 29, 32, 36, 42, 43) and other areas which contain dopamine-sensitive adenylate cyclases (31, 33, 44, 45), and that there are specific brain areas which are particularly rich in the activatable form of phosphodiesterase (5–9). Thus chlorpromazine might cause a fall in cAMP in an area relatively rich in catecholamine-sensitive adenylate cyclases, and a rise in cAMP in areas containing large amounts of activatable phosphodiesterase. Several studies have shown that psychotropic agents can reduce the catecholamine-induced elevation of the concentration of cAMP in brain (26, 28–33, 35, 38). Support for the concept that psychotropic agents can produce a rise in cAMP in the central nervous system comes from a recent report by Palmer *et al.* (46), who demonstrated that injections of amitriptyline and chlorpromazine in mice *in vivo* produced a significant rise in cAMP in the cerebral cortex, an area which is particularly rich in activatable phosphodiesterase (6, 7).

In summary, chlorpromazine and trifluoperazine have been shown to be potent, selective inhibitors of the activation of phosphodiesterase. Unlike EGTA, which inhibits phosphodiesterase activation by chelating calcium, chlorpromazine produces its inhibition by competitively inhibiting the interaction between phosphodiesterase and its activator. This ability of the phenothiazines to inhibit the activation of phosphodiesterase is shared by many other pharmacological agents representing several classes of drugs, each of which is effective in certain forms of psychiatric disorders. That these agents can block an activator-sensitive phosphodiesterase as well as a hormone-sensitive adenylate cyclase might help to explain the anomalous effects of these compounds on the cyclic nucleotide system of brain, which, in turn, might provide insight into their diverse pharmacological actions.

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