MOLECULAR PHARMACOLOGY, 13, 690–697

Binding of Trifluoperazine to the Calcium-Dependent Activator of Cyclic Nucleotide Phosphodiesterase

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(Received December 28, 1976)
(Accepted February 22, 1977)

SUMMARY

LEVIN, ROBERT M. & WEISS, BENJAMIN (1977) Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *Mol. Pharmacol.*, 13, 690–697.

The activity of one form of cyclic nucleotide phosphodiesterase is increased over 10-fold by the addition of an endogenous, calcium-dependent protein activator. This activation of phosphodiesterase is selectively inhibited by phenothiazine antipsychotics such as trifluoperazine. To gain insight into the specific mechanism of this inhibition, we examined the binding of trifluoperazine to various proteins, including the endogenous cyclic nucleotide activator prepared from bovine brain. Using equilibrium dialysis, we found that trifluoperazine binds to proteins at two distinctly different types of sites: a high-affinity, calcium-dependent binding site (K_d = 1 μM, N = 2) and a low-affinity, calcium-independent binding site (K_d = 5 mM, N = 24). All proteins examined, including catalase, cytochrome *c*, aldolase, chymotrypsinogen, egg albumin, bovine serum albumin, and the cyclic nucleotide activator, gave evidence of having low-affinity sites for trifluoperazine. However, only the activator had the calcium-dependent, high-affinity binding sites for the phenothiazine. At low concentrations of trifluoperazine (less than 1 μM), binding to the activator was 10-fold higher in the presence of calcium than in its absence. The binding of trifluoperazine to the other proteins showed no calcium dependence, and the degree of binding to these proteins was similar to the binding of trifluoperazine to activator when measured in the absence of calcium. The binding of trifluoperazine to activator in the presence of calcium markedly decreased when the pH was raised from 7.5 to 8.0, whereas the nonspecific binding was not altered by changes in pH between 6.5 and 8.5. A study of other divalent cations showed that calcium could be replaced by strontium, nickel, cobalt, zinc, and manganese in promoting the binding of trifluoperazine to activator; barium and magnesium were ineffective. These results suggest that the selective inhibition of the activatable form of phosphodiesterase by trifluoperazine is due to specific binding of the phenothiazine to the calcium-dependent protein activator of cyclic nucleotide phosphodiesterase. Since this activator apparently is identical with the activator of adenylate cyclase, our results might also provide the mechanism by which phenothiazines inhibit adenylate cyclase activity and might, in fact, provide a common explanation for some of the diverse physiological and pharmacological actions of the phenothiazine antipsychotics.

This work was supported by Grant CA 15883 from the National Cancer Institute and a grant from the Benevolent Foundation of Scottish Rite Freemasonry, Northern Jurisdiction, U. S. A.

INTRODUCTION

The mammalian brain contains several molecular forms of cyclic nucleotide phos-

ISSN 0026-895X

phodiesterase (1-5). These forms of the enzyme are distributed in the various brain areas in a specific pattern and ratio (3-5), and each has its own peculiar physical and chemical characteristics. The multiple forms of the phosphodiesterases differ in molecular weight (1, 2), substrate specificity (1, 2), stability (1, 3, 4), and response to inhibitors (3, 5, 6) and activators (3-5). One form of phosphodiesterase is of particular interest, since it appears to be the predominant one in most areas of the brain, and its activity can be increased over 10-fold by an endogenous, calcium-dependent protein activator also found in brain (3-5, 7-10).

Recent reports suggest that this same protein activator, which was shown initially to activate phosphodiesterase (7), may also be an important regulator of adenylate cyclase activity (11, 12). Moreover, as in the activation of phosphodiesterase, the response of adenylate cyclase to activator is completely dependent on the presence of calcium. These results suggest that the activator of adenylate cyclase may be identical with the activator of phosphodiesterase.

The activation of phosphodiesterase by this endogenous activator can be selectively inhibited by clinically effective phenothiazine antipsychotic agents, such as chlorpromazine and trifluoperazine (3-6). The inhibition of phosphodiesterase by the phenothiazines can be reversed by increasing the concentration of activator, but not by increasing the concentration of calcium. This suggests that the drugs might act by binding to the activator or to the enzyme rather than to calcium (6).

In order to investigate further the interaction of antipsychotic agents with the cyclic nucleotide system, we have studied the characteristics of binding of trifluoperazine to several proteins, including the purified, calcium-dependent activator prepared from brain.

MATERIALS AND METHODS

Preparation of activator. Activator was purified from bovine brain by the method described by Teo *et al.* (8). Briefly, fresh bovine brains, obtained from a local slaughterhouse, were homogenized in 0.1 M Tris-HCl buffer containing 1 mM Mg^{2+}, pH 7.5. The homogenate was centrifuged at 3000 × *g* for 20 min, and the activator in the supernatant fluid was purified by ammonium sulfate precipitation, DEAE-cellulose anion-exchange chromatography, and column chromatography using Sephadex G-100. Homogeneity of the activator preparation was confirmed by analytical gel electrophoresis; the molecular weight was estimated to be approximately 20,000.

Activator activity. Activator activity was assessed as the ability to increase the activity of an activator-deficient phosphodiesterase prepared from bovine brain (6). One unit of activator is defined as the amount necessary to produce 50% of the maximum activation of this activator-deficient phosphodiesterase attainable under standard experimental conditions, described previously (6). The specific activity of the purified preparation of activator was 100,000 units/mg of protein, which represents approximately a 6000-fold increase over that found in the whole homogenate.

Phosphodiesterase activity. Phosphodiesterase activity was measured by the luciferin-luciferase method as previously described (13). Each reaction vessel contained 50 mM glycylglycine buffer, pH 8.0, 25 mM ammonium acetate, 3 mM $MgCl_2$, 0.18 unit of myokinase, 0.2 unit of pyruvate kinase, 0.4 mM cyclic 3',5'-AMP, 0.1 mM $CaCl_2$, and the phosphodiesterase preparation in a total volume of 160 μl.

Equilibrium dialysis. In general, dialysis baths contained 500 ml of 5 mM Tris-HCl, pH 7.0, containing 1 mM Mg^{2+} and either 0.1 mM Ca^{2+} or 0.3 mM EGTA[1] and various concentrations of [^{3}H]trifluoperazine. Seamless cellulose dialysis tubing (Fisher) having a molecular weight cutoff of 12,000 was filled with 0.5 ml of water, or solutions of activator or other proteins. Dialysis was carried out for 18 hr at room temperature with constant stirring. A portion of the contents of the dialysis membrane and a portion of the bath were

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid; BSA, bovine serum albumin.

counted in a liquid scintillation spectrometer.

During the course of these experiments we observed that the dialysis membrane bound substantial quantities of trifluoperazine, thereby reducing the concentration of free trifluoperazine in the bath. Accordingly, the concentration of free trifluoperazine at the end of the incubation period was used in all calculations. The membranes did not bind any of the proteins studied, nor did the binding of trifluoperazine to the dialysis membrane affect the equilibration of free drug inside and outside the membranes.

Protein. Protein concentration was determined by the method of Lowry *et al.* (14).

Materials. Pyruvate kinase, myokinase, phosphoenolpyruvate, and the purified proteins used in these studies were obtained from Boehringer/Mannheim, and firefly luciferin and luciferase, from du Pont. Trifluoperazine and tritiated trifluoperazine (specific activity, 39.8 mCi/mmole) were generously supplied by Dr. Harry Green of Smith Kline & French Laboratories. Other reagents were obtained from general commercial sources.

RESULTS

Time course of equilibrium between trifluoperazine and activator or BSA. Figure 1A and B shows the rates at which [^{3}H]trifluoperazine added to the bath equilibrated with water, activator, or BSA maintained within dialysis bags. In the bath containing calcium (Fig. 1A) all samples, i.e., water, BSA, and activator, reached equilibrium by 6 hr and remained unchanged for over 20 hr. The concentration of radioactivity in the water and BSA samples at equilibrium was equal to that of the bath, whereas the radioactivity associated with the activator at equilibrium was significantly higher than that associated with either the water or BSA samples. In the bath containing EGTA (Fig. 1B) all samples reached equilibrium by 6 hr and remained constant for over 20 hr. There was only a slight difference between the radioactivity associated with the activator or BSA samples and that of the water samples. After 20 hr of dialysis, calcium was added to the bath containing EGTA to give a final concentration of 0.5 mM free Ca^{2+}. The addition of calcium did not affect the quantity of radioactivity associated with either the water or BSA samples but increased the radioactivity associated with the activator samples to the same level as that of the bath containing calcium but no EGTA (compare Fig. 1A and B).

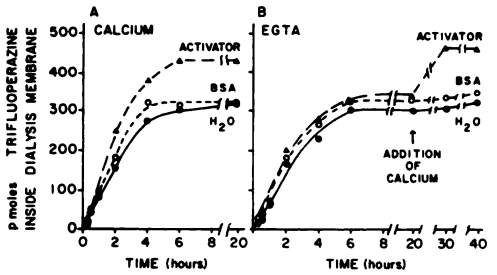

FIG. 1. *Rate of equilibrium between trifluoperazine and activator or BSA*

Each dialysis bath contained 2 liters of 5 mM Tris (pH 7.0), 1 mM Mg^{2+}, and either 0.1 mM Ca^{2+} (A) or 0.3 mM EGTA (B). Dialysis membranes contained 0.5 ml of water, BSA (25 μg/ml), or activator (25 μg/ml). [^{3}H]Trifluoperazine (25 μCi) was added to each bath to give a final concentration of free drug equal to 0.6 μM. At various times, one membrane of each group was removed and the radioactivity within the dialysis membranes was determined in a liquid scintillation spectrometer. After 20 hr calcium chloride was added to the bath in Fig. 1B to give a final concentration of free Ca^{2+} of 0.5 mM.

At the conclusion of the dialysis, we measured the ability of the activator to activate phosphodiesterase. We found only a 10% loss in activator activity over the course of the experiment. These results indicate that trifluoperazine binds to the phosphodiesterase activator and that this binding is dependent on the presence of calcium.

Binding of trifluoperazine as a function of concentration of activator. Figure 2 shows that the binding of trifluoperazine to activator was linear with increasing concentrations of activator when measured in the presence of 0.1 mM calcium or 0.3 mM EGTA. However, the binding of trifluoperazine to activator in the presence of calcium was nearly 6 times greater than in the presence of EGTA.

Effect of cations on binding of trifluoperazine to activator and BSA. Figure 3 shows the influence of several divalent cations on the binding of trifluoperazine to the cyclic nucleotide activator or to BSA.

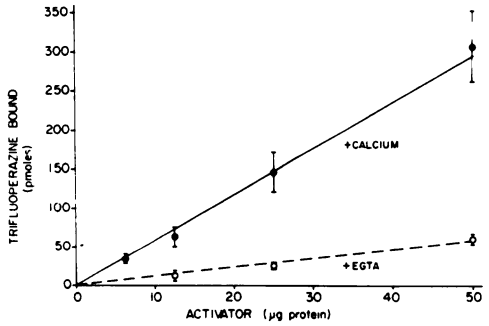

FIG. 2. *Binding of trifluoperazine as a function of concentration of activator*

Each dialysis bath contained 2 liters of 5 mM Tris (pH 7.0), 1 mM Mg^{2+}, and either 0.1 mM Ca^{2+} or 0.3 mM EGTA. The dialysis membranes contained either water or different concentrations of activator. [^{3}H]Trifluoperazine (8 μCi) was added to the bath to give a final concentration of free trifluoperazine of 0.2 μM. The samples were dialyzed at room temperature for 18 hr, after which time the contents were removed and radioactivity was determined in a liquid scintillation spectrometer. Each point is the mean value of four samples. Vertical brackets indicate standard errors.

In the absence of any divalent cation, trifluoperazine was equally bound to activator and BSA. At the concentrations used, none of the divalent cations examined affected significantly the binding of trifluoperazine to BSA. However, several ions increased the binding to activator: calcium was the most effective, increasing the binding to activator over 9-fold; strontium, nickel, zinc, and manganese also promoted the binding of trifluoperazine to activator, but barium and magnesium failed to alter the binding at these concentrations.

Binding of trifluoperazine to various proteins. Figure 4 shows the influence of calcium on the binding of trifluoperazine to activator and several other proteins with molecular weights ranging from 12,000 to 240,000. Whereas calcium markedly increased the binding of trifluoperazine to activator, it failed to increase the binding of the phenothiazine to any of the other proteins examined.

Effect of pH on binding of trifluoperazine to activator and BSA. The binding of trifluoperazine to activator in the presence of EGTA was unaffected by changing the pH from 6.5 to 8.5 (Fig. 5). However, there was a marked reduction in the calcium-

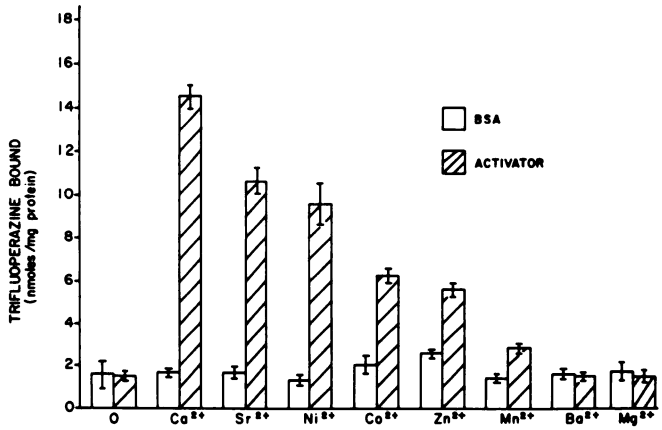

FIG. 3. *Effects of various divalent cations on binding of trifluoperazine to activator and BSA*

Dialysis baths contained 0.5 liters of 5 mM Tris (pH 7.0), 1 mM Mg^{2+}, 0.1 mM EGTA, and a 0.3 mM concentration of one of the following salts in the divalent form: calcium chloride, magnesium chloride, zinc chloride, cobalt chloride, strontium chloride, nickel chloride, barium chloride, and manganese chloride. The dialysis membranes contained either 0.5 ml of water, BSA (20 μg/ml), or activator (20 μg/ml). [^{3}H]Trifluoperazine (8 μCi) was added to the baths to give a final concentration of free trifluoperazine of 0.2 μM. The samples were dialyzed for 18 hr, after which time radioactivity was determined. Each value is the mean of three samples. Vertical brackets indicate standard errors.

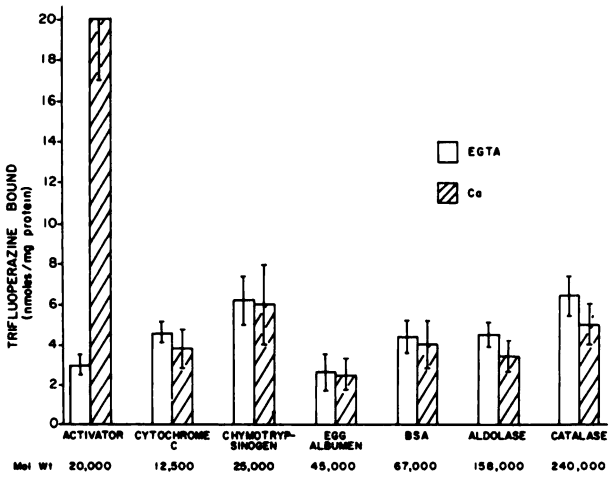

FIG. 4. *Binding of trifluoperazine to various proteins*

The dialysis baths contained 2 liters of 5 mM Tris (pH 7.0), 1 mM Mg^{2+}, and either 0.1 mM Ca^{2+} or 0.3 mM EGTA. The dialysis membranes contained 0.5 ml of each of the following proteins at a concentration of 50 μg/ml: cytochrome *c* (mol wt 12,500), activator (20,000), chymotrypsinogen (25,000), egg albumin (45,000), bovine serum albumin (67,000), aldolase (158,000), and catalase (240,000). [^{3}H]Trifluoperazine (8 μCi) was added to give a final concentration of free trifluoperazine of 0.2 μM. The samples were dialyzed for 18 hr, after which time radioactivity was determined. Each value represents the mean of three samples. Vertical brackets indicate standard errors.

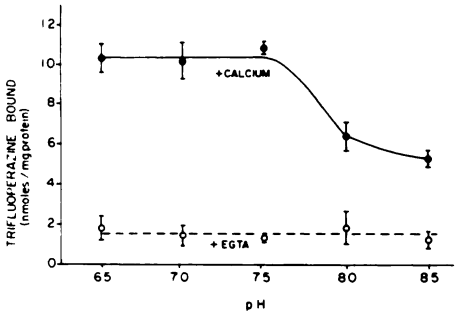

FIG. 5. *Effect of pH on binding of trifluoperazine to activator and BSA*

The dialysis baths contained 0.5 liters of 5 mM Tris at various pH values, 1 mM Mg^{2+}, and either 0.1 mM Ca^{2+} or 0.3 mM EGTA. The dialysis membranes contained 0.5 ml of water, BSA (20 μg/ml), or activator (20 μg/ml). [^{3}H]Trifluoperazine (12 μCi) was added to give a final concentration of 0.3 mM. Samples were dialyzed for 18 hr, after which time radioactivity was determined. Each point represents the mean of three samples. Vertical brackets indicate standard errors.

specific binding of trifluoperazine to activator above pH 7.5.

The binding of trifluoperazine to BSA in the presence of calcium or EGTA was unaffected by changes in pH and was equal to the binding of the phenothiazine to activator in the presence of EGTA (data not shown).

Binding of trifluoperazine to activator and BSA as a function of concentration of trifluoperazine. Significant calcium-specific binding was evident at concentrations of free trifluoperazine as low as 0.002 μM (Fig. 6A). The ratio of calcium-specific to calcium-independent binding progressively decreased until, at concentrations of trifluoperazine above 100 μM, there was no significant difference between binding in the presence and absence of calcium. The binding of trifluoperazine to BSA in the presence and absence of calcium was similar to the binding of trifluoperazine to activator in the absence of calcium (Fig. 6B).

The data on the binding of trifluoperazine to activator in the presence of calcium were plotted according to the Scatchard equation (Fig. 7). Two types of binding sites were revealed: high-affinity, low-capacity binding with a dissociation constant for trifluoperazine (K_d) of 1 μM and two binding sites per molecule (based on a molecular weight of 20,000 for activator), and low-affinity, high-capacity binding with a

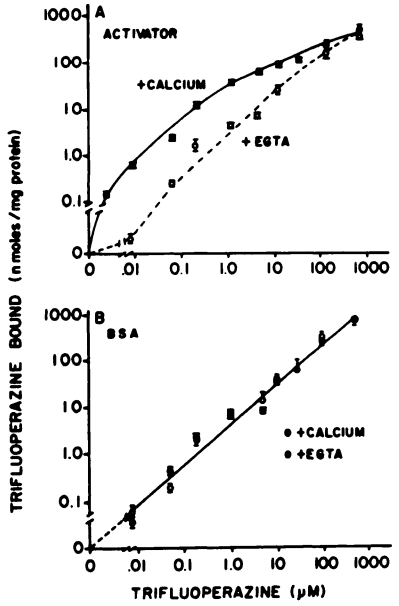

FIG. 6. *Binding of trifluoperazine to activator and BSA as a function of concentration of trifluoperazine*

The dialysis baths contained 0.5 liters of 5 mM Tris (pH 7.0), 1 mM Mg^{2+}, and either 0.1 mM Ca^{2+} or 0.3 mM EGTA. The dialysis membranes contained 0.5 ml of water, activator (25 μg/ml) (A), or BSA (25 μg/ml) (B). [^{3}H] Trifluoperazine and unlabeled trifluoperazine were added to the baths to give final concentrations of free drug ranging from 0.002 to 500 μM. Samples were dialyzed for 18 hr, after which time radioactivity was determined. Each point represents the mean value of three samples. Vertical brackets indicate standard errors.

K_d of 5 mM and 24 binding sites per molecule.

The binding of trifluoperazine to activator when measured in the presence of EGTA or the binding of trifluoperazine to BSA when measured in the presence of either calcium or EGTA showed only the low-affinity, high-capacity sites.

DISCUSSION

Despite the long and successful use of phenothiazine antipsychotics in the treatment of certain forms of mental disease, the mechanism by which these agents act is still poorly understood. One current hypothesis that has received extensive review recently is that these compounds act by interfering with the cyclic nucleotide system of brain (15, 16). The action of these compounds, however, is complex, in that antipsychotics have been shown to inhibit specific forms of both adenylate cyclase (17–21) and phosphodiesterase (3–6). Recent evidence showing that a heat-stable, calcium-binding protein can activate both these enzyme systems (3–6, 11, 12) suggests that phenothiazines may inhibit these enzymes by a common mechanism, namely, by binding to this protein activator.

Our results show that trifluoperazine does bind to this activator protein at two types of binding sites: (a) calcium-dependent binding which is specific and saturable, with a dissociation constant of approximately 1 μM and apparently two binding sites for trifluoperazine per molecule, and (b) calcium-independent binding which has a dissociation constant of 5 mM and about 24 sites per molecule. Of several other proteins examined, only the activator possessed the high-affinity, calcium-dependent sites; the other proteins had the low-affinity, calcium-independent sites only.

The specific binding of trifluoperazine to activator can be affected not only by calcium but by other divalent cations as well. Calcium can be replaced by strontium, nickel, zinc, and manganese, but not by magnesium or barium. In general, the

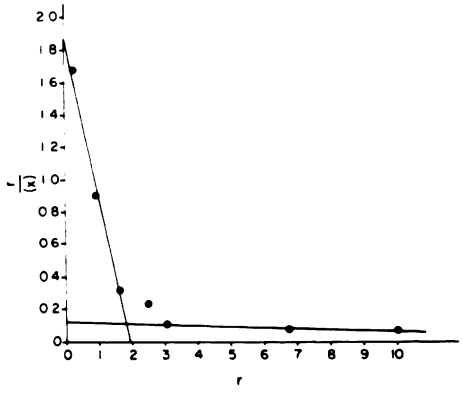

FIG. 7. *Scatchard plot of binding of trifluoperazine to activator*

The data obtained in Fig. 6A were plotted using the Scatchard equation, where r = moles of trifluoperazine bound per mole of activator and (x) = concentration of free trifluoperazine at equilibrium.

same ions which can replace calcium in the binding of trifluoperazine to activator can also replace calcium in the activation of phosphodiesterase (9).

These results suggest that phenothiazines inhibit the activation of phosphodiesterase by selectively binding to the calcium-dependent phosphodiesterase activator. This suggestion is supported by the evidence showing that the activation of phosphodiesterase and the binding of trifluoperazine to activator are calcium-dependent and that several other ions can replace calcium for the activation of phosphodiesterase (9) as well as for the binding of trifluoperazine to activator (Fig. 3). A comparison of the K_d value for the specific binding of trifluoperazine to activator and the K_i (5) or I_{50} values (6) for the inhibition of the activation of phosphodiesterase by trifluoperazine shows that the I_{50} and K_i values for phosphodiesterase inhibition were about 10 times greater than the K_d values for binding. It should be noted, however, that the I_{50} and K_i values are dependent upon the concentration of activator present. In these studies supramaximal amounts of activator were added. When the experiments are performed with subsaturating concentrations of activator, considerably lower I_{50} and K_i values result.[2] That the binding of trifluoperazine to activator constitutes the mechanism by which this drug inhibits the activation of phosphodiesterase is also supported by other recent observations (22). The antipsychotic agents pimozide and penfluridol, which are potent antagonists of the activation of phosphodiesterase (6), also show calcium-specific binding to the activator. On the other hand, the benzodiazepine chlordiazepoxide, which is relatively ineffective in blocking the activation of phosphodiesterase (6), failed to exhibit calcium-specific binding to the activator.

The studies on the influence of pH on binding and phosphodiesterase activation are difficult to interpret, since the activity of phosphodiesterase itself, as well as its activation, is altered by pH. A change in pH, which may alter the binding of the activator to the enzyme, may be unrelated to the binding of trifluoperazine to activator. The data showing that the binding of trifluoperazine to activator markedly decreased at pH values above 7.5, however, do suggest that the binding sites on the activator molecule may be altered at these pH values.

Whether trifluoperazine and phosphodiesterase bind to the same sites on the activator is still an open question. Similar concentrations of calcium are required for the activation of phosphodiesterase and for the binding of trifluoperazine to activator (8, 23). Moreover, there are two high-affinity binding sites per molecule of activator for both calcium (23) and trifluoperazine, and the association constants of the activator for calcium and trifluoperazine are similar (23).

Our results showing the specific binding of trifluoperazine to the cyclic nucleotide activator may help to explain the opposing actions of phenothiazines on the cyclic nucleotide system. On the one hand, these agents block the elevation of cyclic AMP in specific brain areas induced by decapitation (17), norepinephrine (17, 18), and dopamine (19–21); on the other, they increase the intracellular concentration of cyclic AMP (24, 25) and cyclic GMP (26) in certain brain areas. These effects of phenothiazines are not contradictory if one considers the complexity of the cyclic nucleotide system in the central nervous system. Both the nucleotide cyclases and the cyclic nucleotide phosphodiesterases exist in several molecular forms, and different brain areas have different patterns and ratios of these enzymes. Since phenothiazine antipsychotics specifically inhibit the catecholamine-sensitive adenylate cyclase and the activator-sensitive cyclic nucleotide phosphodiesterase, the net effect of these antipsychotics on the cyclic nucleotide concentration in each specific region of the brain would depend on the relative activities of the specific forms of each of these enzyme systems. In an area rich in activator-sensitive phosphodiesterase, phenothiazines would be expected to increase the intracellular concentration of cyclic AMP or cyclic GMP, whereas in an area rich in catecholamine-sensitive adenylate cyclase, pheno-

[2] R. M. Levin and B. Weiss, unpublished observations.

thiazines would produce a decrease in cyclic AMP.

The significance of the same protein activator influencing both adenylate cyclase and phosphodiesterase has been discussed by Gnegy *et al.* (27). They demonstrated that membrane-bound activator can be released from particulate preparations of brain by a cyclic AMP-dependent protein kinase. Accordingly, the stimulation of adenylate cyclase would raise the intracellular concentration of cyclic AMP, which in turn could cause the release of membrane-bound activator by a cyclic AMP-dependent protein kinase. The release of bound activator could then stimulate the activator-sensitive phosphodiesterase, thereby causing the hydrolysis of cyclic AMP.

In conclusion, the evidence that the cyclic nucleotide activator modulates the activity of both phosphodiesterase and adenylate cyclase as well as our demonstration that trifluoperazine and other antipsychotic agents (22) bind in a highly specific fashion to this activator, suggests a common mechanism for explaining the inhibition by antipsychotics of selective forms of these enzymes and a common mechanism for explaining some of their diverse pharmacological effects.

ACKNOWLEDGMENTS

We acknowledge with thanks the excellent technical assistance of Mr. Thomas Wallace.

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