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Mechanism of Phenothiazine Inhibition of Ca^{2+}-dependent Guanosine 3',5'-(cyclic) Monophosphate Phosphodiesterase of Brain

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SUMMARY

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Trifluoperazine inhibition of a partially purified cerebral cortical phosphodiesterase having a high stimulatory response to a Ca^{2+}-dependent regulatory (CDR) protein and specificity toward guanosine 3',5'(cyclic) monophosphate was studied. Trifluoperazine (50 μM) completely blocked the Ca^{2+}·CDR-stimulated activity but had no effect on the basal activity. The magnitude of the inhibition depended on the concentrations of both inhibitor and activator. With increasing concentration of CDR, the enzyme became less sensitive to the phenothiazine. Plots of 1/V vs 1/CDR and 1/V vs trifluoperazine concentration were nonlinear with upward curvature, in contrast to a previous report. Decreasing the pH of the reaction mixture from 8.0 to 6.8 enhanced inhibition while reducing the K_m for cyclic GMP and produced no change in affinity of the enzyme for Ca^{2+}·CDR. These results correlate well with previous reports on Ca^{2+}·CDR-phenothiazine binding and provide further support for a mechanism in which trifluoperazine, upon binding to Ca^{2+}·CDR, renders the latter ineffective in stimulating the enzyme, thus depleting the level of active CDR and inhibiting the activator-dependent phosphodiesterase. This reduction in active CDR may be involved in a mode of phenothiazine action on the activities of other Ca^{2+}·CDR-dependent enzymes.

INTRODUCTION

Several antipsychotic drugs, *e.g.*, the phenothiazines, have been shown to inhibit a specific brain cyclic nucleotide phosphodiesterase, (an enzyme) characterized by stimulation by Ca^{2+} and a Ca^{2+}-dependent regulatory (CDR)[1] protein (1). Only the stimulated activity was inhibited; the basal activity of the enzyme was unaffected. Since the identical CDR protein is also involved in the activation of brain adenylate cyclases (2-4), protein kinases (5-8), and (Ca^{2+} + Mg^{2+}) ATPase (9, 10), as well as the relief of troponin-I inhibition of actomyosin ATPase (11), and thus appears to have a multifunctional role as a mediator of various Ca^{2+} regulatory signals, studies on how drugs interact with the phosphodiesterase-CDR complex take on added importance.

The mechanism by which phenothiazines inhibit Ca^{2+}·CDR activated cerebral phosphodiesterase has not been clearly established. Kinetic analyses of chlorpromazine and trifluoperazine inhibition of hydrolysis

[1] The abbreviations used are: CDR, Ca^{2+}-dependent regulator; cyclic AMP, adenosine cyclic 3',5'-monophosphate; cyclic GMP, guanosine cyclic 3',5'-monophosphate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetraacetic acid.

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of 400 μM cyclic AMP seemed to indicate competitive interaction between CDR and the phenothiazines (1) at the Ca^{2+}·CDR binding site on the enzyme (see Webb [12]). However, subsequent studies with radioactive inhibitors revealed high affinity, pH and Ca^{2+}-dependent, CDR-specific binding that correlated with the potency of these agents to inhibit the Ca^{2+}·CDR-stimulated cyclic AMP phosphodiesterase (13–15). These results led to the suggestion that binding to CDR was responsible for inhibition of the phosphodiesterase (13–15). Our previous studies of brain cyclic nucleotide phosphodiesterases indicated that at physiological substrate concentrations, 1 μM, the preferred substrate for the Ca^{2+}·CDR-dependent enzyme was cyclic GMP (16). Moreover, the phosphodiesterase preparation was activated up to 40-fold by Ca^{2+}·CDR (16), whereas the early preparations had considerably smaller activation (1, 13). Thus, the apparent kinetic inconsistency, the availability of a preparation of phosphodiesterase exhibiting high responsiveness to activator, and the use of cyclic GMP as substrate led us to re-examine the mechanism of the phenothiazine inhibition. These findings, which are presented in this paper, provide kinetic support for the suggested mechanism of inhibition whereby trifluoperazine binding to Ca^{2+}·CDR prevents the activation of the Ca^{2+}·CDR-dependent enzyme.

MATERIALS AND METHODS

[8-^{3}H]Cyclic AMP (21–30 Ci/mmol), [8-^{3}H]cyclic GMP (19–21 Ci/mmol), [^{14}C]-adenosine (464 mCi/mmol), and [^{14}C]guanosine (585 mCi/mmol) were purchased from Amersham-Searle. Cyclic AMP was obtained from P-L Biochemicals. Cyclic GMP, neutral alumina, and 5′-nucleotidase were from Sigma. Chlorpromazine and trifluoperazine were from Smith, Kline, and French Laboratories, Philadelphia.

Preparation of phosphodiesterase and CDR. The Ca^{2+}·CDR-dependent phosphodiesterase and partially purified CDR were prepared from pig cerebral cortex as previously described (16). Briefly, a cortical extract was chromatographed on DEAE-cellulose with 20 mM Tris, pH 7.5, 1 mM EGTA and a linear 0–0.4 M NaCl gradient. The cyclic GMP specific, Ca^{2+}·CDR-dependent phosphodiesterase, which eluted at approximately 0.18 M NaCl, was rechromatographed in an identical manner and the enzyme eluted in the same position. Selected fractions from this peak, termed D-I in accordance with the nomenclature of Russell *et al.* (17), were combined and dialyzed against 20 mM Tris-maleate-HCl, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, then stored at −50° after addition of bovine serum albumin to 0.1%.

CDR was eluted from the DEAE-cellulose column at approximately 0.28 M NaCl. After heating for 3 min at 95–100° the CDR-containing fractions were centrifuged at 20,000 × *g* for 10 min, the supernatants were combined and dialyzed against 20 mM Tris-maleate, pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, and stored at −50°.

Assays. Phosphodiesterase was assayed as previously described (16). Reaction mixtures contained 20 mM Tris-maleate-HCl, pH 8.0, unless otherwise indicated, 5 mM $MgCl_2$, 100 mM KCl, 1.0 mM EGTA, 1.2 mM $CaCl_2$ when present, 0.1% bovine serum albumin, 20–40 pmol [^{3}H]cyclic nucleotide plus added, unlabeled cyclic nucleotide to obtain the appropriate concentration, and enzyme plus or minus CDR and inhibitors in a total volume of 100 μl. Following incubation at 20° for 15–120 min, the reaction was stopped by addition of 50 μl of 0.8 N perchloric acid and the mixture subsequently neutralized with 50 μl of 0.8 N KOH containing 0.20 M Tris-Cl previously adjusted to pH 8.2. All subsequent steps and calculations were performed as previously described (16). Under the conditions of assay, activities in the presence or absence of CDR and/or phenothiazine were proportional to time and amount of enzyme. All data shown are the means of duplicate or triplicate determinations, with all values within 10%, usually 2–5%, of the mean.

Protein was assayed by the method of Lowry *et al.* (18), with bovine serum albumin as the standard protein.

RESULTS

Inhibition of the Ca^{2+}·CDR-dependent phosphodiesterase. The effect of trifluoper-

azine on the rates of hydrolysis of cyclic nucleotides by the D-I phosphodiesterase is shown in Table 1. In the absence of Ca^{2+}·CDR, the D-I enzyme hydrolyzed cyclic GMP at a rate 7-times that of cyclic AMP, when the concentration of each substrate was 1 μM. Trifluoperazine (50 μM) had essentially no effect on these basal activities. In the presence of Ca^{2+}·CDR, phosphodiesterase activities were increased about 40 times and the phenothiazine virtually completely inhibited the stimulated activity, with either cyclic GMP or cyclic AMP as substrate. Increasing the Ca^{2+} concentration to 2.0 mM (1.0 mM free Ca^{2+}) neither reversed nor modified the inhibition (data not shown), indicating that the trifluoperazine effect was not due to chelation of Ca^{2+}. These findings are in agreement with previous results (1, 16).

Effect of the concentration of CDR on the inhibition by trifluoperazine. The effect of various concentrations of CDR on the action of different concentrations of trifluoperazine is described in Figure 1. With increasing concentration of CDR, the enzyme became less sensitive to the phenothiazine. This decreased sensitivity occurred over a relatively narrow range of CDR concentration; for example, with 8 μM trifluoperazine, the inhibition was decreased 90% by a 5-fold increase in the activator concentration.

Kinetic analyses of the trifluoperazine-CDR interaction are shown in Figure 2. A plot of the reciprocal of the rate of phosphodiesterase activity as a function of the reciprocal of CDR, at different trifluoperazine concentrations, resulted in a series of nonlinear lines with upward curvature (Fig. 2A). These findings with a highly responsive enzyme and with cyclic GMP as substrate are in marked contrast with the previously reported linear relationship (1). To be noted, in these experiments (Fig. 2) the cyclic GMP concentration was increased to 5 μM to minimize the effect of Ca^{2+}·CDR on the K_m of the enzyme (16; see Fig. 5, this paper); thus under these conditions Ca^{2+}·CDR behaved as a nonessential activator. Dixon plots of the reciprocal of the rate as a function of the concentration of trifluoperazine, at different CDR concentrations, were similarly curvilinear (Fig. 2B). These results are inconsistent with phenothiazine inhibition by competition with Ca^{2+}·CDR for the activator site on the phosphodiesterase (1), but are indicative of the depletion of the level of active CDR.

Effect of pH on the trifluoperazine inhibition of phosphodiesterase activity. The effect of pH on inhibition of the stimulated phosphodiesterase is shown in the experiment described in Fig. 3. When the pH of the reaction mixture was lowered from 8.0 to 6.8, the dose response curve for trifluoperazine shifted to the left, resulting in a

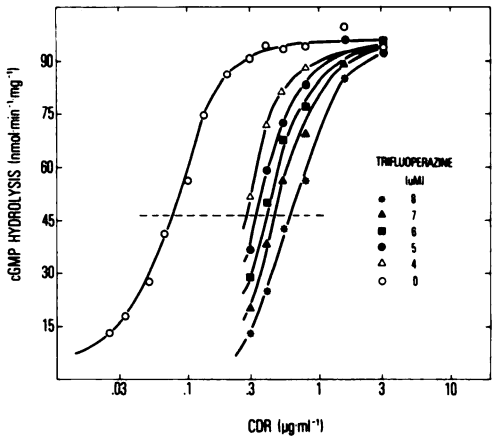

FIG. 1. *The effect of various concentrations of CDR on the inhibition of the D-I phosphodiesterase by different concentrations of trifluoperazine*

Incubation conditions were as described in the text with cyclic GMP at 5 μM, $CaCl_2$ at 1.2 mM, and EGTA at 1.0 mM. The dashed line represents 50% of the maximal activity obtained with saturating CDR.

TABLE 1

Effect of trifluoperazine on cyclic nucleotide phosphodiesterase (D-I) activity of cerebral cortex

Activities were measured with either cyclic GMP or cyclic AMP at 1 μM. All assays contained 1.2 mM $CaCl_2$ and 1.0 mM EGTA. Partially purified CDR was present, where indicated, at 0.32 $\mu g \cdot ml^{-1}$.

Trifluoperazine	CDR	Phosphodiesterase activity	
		Cyclic GMP	Cyclic AMP
(50 μM)		*($nmol \cdot min^{-1} \cdot mg^{-1}$)*	
−	−	1.52	0.27
+	−	1.56	0.25
−	+	67.1	10.7
+	+	2.7	0.21

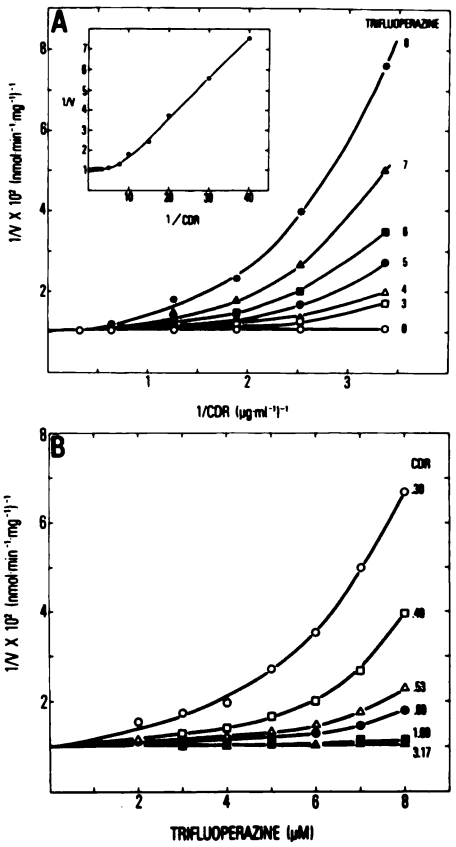

FIG. 2. *Kinetic analyses of the trifluoperazine-CDR interaction*

Values were calculated from the data illustrated in Fig. 1. In (A), a Lineweaver-Burk transformation of the rates as a function of CDR concentration is shown for different trifluoperazine concentrations. The inset shows the effect of CDR over a broader range of concentrations (0.025 to 3.17 $\mu g \cdot ml^{-1}$), in the absence of the phenothiazine. In (B), a Dixon plot of the rates as a function of trifluoperazine concentration is shown for different CDR concentrations.

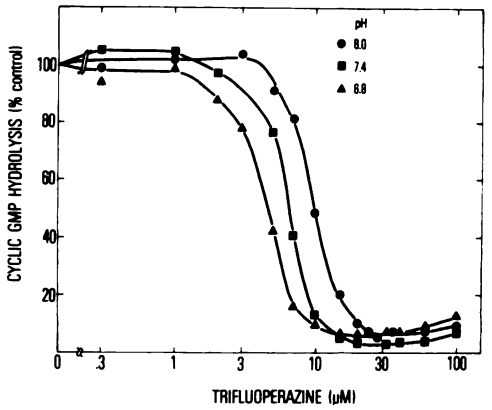

FIG. 3. *Effect of pH on the trifluoperazine inhibition of Ca^{2+}·CDR-stimulated phosphodiesterase activity*

Incubation conditions were as described in the text with cyclic GMP at 1 μM and CDR at 0.32 $\mu g \cdot ml^{-1}$.

decrease in the IC_{50} value of approximately 50%. A similar pH dependence was found with chlorpromazine, but the IC_{50} values were 2- to 3-times greater than with trifluoperazine (data not shown).

It was feasible that this observed increase in sensitivity of the enzyme to trifluoperazine with lowered pH was due to (a) a pH-dependent reduction in the sensitivity of the D-I phosphodiesterase to Ca^{2+}·CDR, rather than to a pH-dependent interaction of Ca^{2+}·CDR with inhibitor, or (b) an increase in the K_m for cyclic GMP permitting trifluoperazine inhibition of substrate binding. The results in Figs. 4 and 5 rule out both possibilities. Dose-response curves of phosphodiesterase activation as a function of Ca^{2+}·CDR concentration at different pH values (Fig. 4) did indicate a slight shift in the "K_a" for CDR, but in the direction of even greater sensitivity. Although lowering the pH from 8.0 to 6.8 did substantially reduce the V_{max}, the K_m was also decreased (Fig. 5), suggesting even greater affinity of the enzyme for cyclic GMP at the lower pH. Therefore, these findings are consistent with previous reports of increased binding of trifluoperazine to Ca^{2+}·CDR at the lower pH (13) and with the suggestion that phenothiazines, upon binding to Ca^{2+}·CDR, effect inhibition of phosphodiesterase activity by reversing the Ca^{2+}-conferred stimulatory potency and depleting the level of active CDR (13–15).

DISCUSSION

The overall effects of the phenothiazines on cyclic nucleotide metabolism are likely to be complex and tissue specific, a consequence of variable degrees of inhibition of both synthesis of cyclic AMP (3, 19) and degradation of cyclic AMP and cyclic GMP. Cerebral Ca^{2+}·CDR-dependent phosphodiesterase has strong specificity for cyclic GMP at physiological substrate concentrations (16; Table 1, this paper). The ratio of

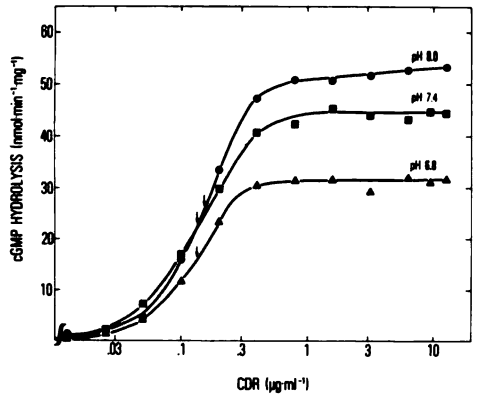

FIG. 4. *Effect of pH on the activation of D-I phosphodiesterase by CDR*

The cyclic GMP concentration was 1 μM. Arrows indicate the concentrations of CDR resulting in 50% activation.

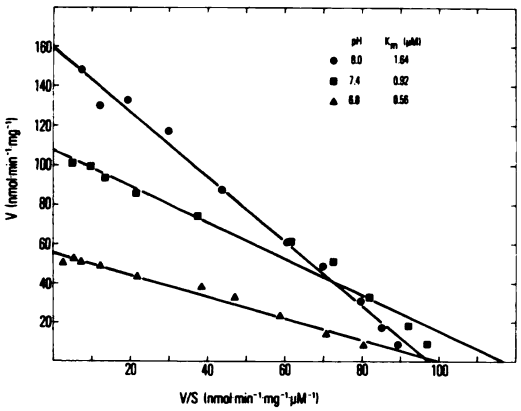

FIG. 5. *Effect of pH on the kinetics of the Ca^{2+}·CDR-stimulated phosphodiesterase with respect to cyclic GMP*

The concentration of CDR was 0.32 $\mu g \cdot ml^{-1}$.

the concentrations of cyclic AMP/cyclic GMP in neural tissues may approach or even drop below unity (20, 21). Both factors explain why phenothiazine inhibition of cyclic nucleotide hydrolysis should have greater effects on the level of cyclic GMP than on that of cyclic AMP. Additionally, hydrolysis of cyclic AMP by a different cerebral phosphodiesterase, which was found to be stimulated by cyclic GMP (16), would tend to shift the cyclic nucleotide concentration ratio to even lower values. Inhibition of the Ca^{2+}·CDR-dependent adenylate cyclase would also lead to a decrease in the ratio.

The phenothiazines are known to inhibit both the cerebral cortical adenylate cyclase dependent on Ca^{2+}·CDR (3) and the striatal cyclase stimulated by dopamine (19). Dopamine sensitivity of the striatal enzyme was subsequently found to correlate with the level of membrane-associated CDR (4). That phenothiazine inhibition of dopamine-stimulated cyclase results from phenothiazine-Ca^{2+}·CDR binding is uncertain, however, since dopamine activation and phenothiazine inhibition were observed in the presence of EGTA (19). The effects of micromolar free Ca^{2+}, buffered by EGTA, in the presence and absence of CDR, on this Ca^{2+}·CDR associated, dopamine stimulated adenylate cyclase have not been reported and this information is needed to understand better this inhibitory effect of phenothiazines.

Assessment of the significance of phenothiazine-Ca^{2+}·CDR binding to inhibition of dopamine-stimulated adenylate cyclase is further complicated. The K_d value of 1 μM for trifluoperazine binding to Ca^{2+}·CDR (13) is substantially higher than the K_i or IC_{50} values found for phenothiazine inhibition of both the cyclase (19) and the binding of antagonists to dopamine receptors (22). The high membrane/buffer partitioning of phenothiazines (23), however, strongly suggests that membrane-associated CDR may indeed be a site of action. It is noteworthy that CDR is present in substantial levels in membranes (4, 24, 25), along with Ca^{2+}·CDR-dependent enzymes (2, 5, 9, 10), and binds to neural membranes in a Ca^{2+}-dependent, reversible manner (25). Thus, it is possible that phenothiazines, by reversing the stimulatory potency of Ca^{2+}·CDR, inhibit a number of enzymes, particularly those that are membrane-associated, in a manner analogous to that shown for the cyclic GMP phosphodiesterase, and exert a general negative influence on intracellular Ca^{2+} regulatory signals.

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