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EFFECTS OF PHENOTHIAZINES ON THE MEMBRANE-BOUND GUANYLATE AND ADENYLATE CYCLASE IN *TETRAHYMENA PYRIFORMIS*

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Abstract—The particulate-bound guanylate cyclase activity of *Tetrahymena pyriformis* was shown previously to be Ca^{2+}-dependent and to be activated by an endogenous calmodulin-like protein (*Tetrahymena* Ca^{2+}-binding protein, TCBP) [S. Nagao, Y. Suzuki, Y. Watanabe and Y. Nozawa, *Biochem. biophys. Res. Commun.* **90**, 261 (1979)]. Phenothiazine derivatives, such as chlorpromazine and trifluoperazine, that interact with calmodulin were found to inhibit the Ca^{2+}-dependent guanylate cyclase activity and the TCBP-induced activation of the guanylate cyclase activity. Ethylene glycol-bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), a Ca^{2+} chelator, also inhibited the activation of guanylate cyclase. However, the mechanisms by which EGTA and trifluoperazine act were different. The EGTA-induced inhibition could not be overcome by increasing the concentration of TCBP, whereas the trifluoperazine-induced inhibition could be overcome by increasing the concentration of TCBP, but not by increasing the concentration of Ca^{2+}. These findings suggest that the mechanism by which trifluoperazine inhibits the activation of guanylate cyclase involves competition with TCBP.

Recent studies have shown that calmodulin exists ubiquitously in eukaryotic cells as a generalized Ca^{2+} target protein and regulates a wide variety of enzyme systems [for review see Refs. 1–4]. The enzymes regulated by calmodulin include a cyclic nucleotide phosphodiesterase [5, 6], an adenylate cyclase [7], a myosin light chain kinase [8–10], a membrane ATPase [11–13], a phosphorylase kinase [14], and an NAD kinase [15]. In a previous report [16], we described that guanylate cyclase (EC 4.6.1.2) activity in *Tetrahymena pyriformis* is entirely associated with particulate fractions, but not with soluble fractions, and presented evidence that the enzyme activity is Ca^{2+}-dependent and is activated by an endogenous activator protein (TCBP)† in the presence of low concentrations of Ca^{2+}. This activator protein then turned out to be a Ca^{2+}-binding protein of acidic and thermostable nature and, therefore, its possible identification as calmodulin was suggested [17, 18].

Phenothiazine psychotropic agents such as trifluoperazine have been used as a tool to study the mechanism of action of calmodulin since they bind to this protein [19] in the presence of Ca^{2+}, preventing the stimulation of cyclic nucleotide phosphodiesterase [19], adenylate cyclase [20], phosphorylase kinase [21], and erythrocyte Ca^{2+}–Mg^{2+} ATPase [22] by added calmodulin. These findings prompted us to investigate effects of phenothiazine derivatives on the activity of *Tetrahymena* guanylate cyclase.

We now report that the drugs also inhibit *Tetrahymena* guanylate cyclase activity and suggest that the drug-induced inhibition of the enzyme would be caused by interfering with its activation by TCBP.

MATERIALS AND METHODS

Materials. [8-^{3}H]GTP and [2-^{3}H]ATP were purchased from the Radiochemical Centre, Amersham, U.K. All unlabeled nucleotides used were obtained from the Sigma Chemical Co., St. Louis, MO. Creatine phosphate and creatine kinase were purchased from Boehringer Mannheim, West Germany. 3-Isobutyl-1-methylxanthine was obtained from the Aldrich Chemical Co., Milwaukee, WI. Neutral aluminum oxide was a product of Woelm Pharma, West Germany. Chlorpromazine, propericiazine and trifluoperazine were supplied by the Yoshitomi Pharmaceutical Co., Osaka, Japan.

Preparation of Tetrahymena Ca^{2+}*-binding protein.* *Tetrahymena* Ca^{2+}-binding protein was prepared by the method described by Suzuki *et al.* [17], with slight modifications. Acetone-dried powder was prepared by washing *Tetrahymena* cells three times with acetone and extracted with 10 vol. of distilled water for 30 min at 95°. The supernatant fluid was fractionated with solid ammonium sulfate between 60 and 80% saturation. The precipitate was redissolved in a minimum of 10 mM Tris–HCl, 0.5 mM EGTA (pH 7.5) and dialyzed against the same buffer. The dialyzed protein solution was applied to a column of DEAE-cellulose equilibrated with the above buffer, and the unbound material was washed through with 500 ml of buffer. The column was then

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† Abbreviations: TCBP, *Tetrahymena* Ca^{2+}-binding protein; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid; cyclic GMP, guanosine 3′,5′-monophosphate; and cyclic AMP, adenosine 3′,5′-monophosphate.

eluted with 10 mM Tris–HCl (pH 7.5) containing increasing concentrations of KCl (several bed volumes each of 0.20 and 0.40 M). Under these conditions, TCBP usually appeared in the 0.40 M KCl eluate. Fractions containing the TCBP were used for further purification by preparative electrophoresis on polyacrylamide gels in the presence of 40% glycerol (pH 8.6) [23]. When the final preparation obtained after several purification steps was subjected to electrophoresis on polyacryamide gels in the presence of 40% glycerol or 0.1% sodium dodecylsulfate (pH 7.2) [24], the protein sample (15 μg) was single banded.

Enzyme preparation. A thermotolerant strain of *T. pyriformis* (NT-1) was grown at 39.5° in an enriched proteose–peptone medium as described previously [25]. Cultures of 200 ml were harvested in the early stationary phase of growth. The cell suspension (5×10^8 cells/100 ml) in 10 mM Tris–HCl buffer (pH 7.5), containing 250 mM sucrose, 1 mM dithiothreitol and 0.5 mM EGTA, was sonicated at 9 kilocycles for 2 min by a Branson Sonifier (B-12) to obtain the homogenate. The homogenate was centrifuged at 105,000 *g* for 60 min and the pellet was washed by resuspension and homogenization in 20 vol. of the sonicating fluid. The homogenization and centrifugation procedure was performed twice. The final pellet was resuspended and rehomogenized in the same Tris–HCl buffer without EGTA to give appropriate protein concentrations and, then, was used for enzyme assays.

Guanylate and adenylate cyclase assay. Unless otherwise indicated, the standard assay mixture for guanylate cyclase contained 1 mM [^{3}H]GTP (5 Ci/mole), 1 mM cyclic GMP, 15 mM creatine phosphate, 20 μg creatine kinase, 1 mM dithiothreitol, 1.5 mM isobutylmethylxanthine, 3 mM $MgCl_2$, 100 μM $CaCl_2$, 25 mM Tris–maleate (pH 6.8) and 100 μg of enzyme protein in a total volume of 0.20 ml. For the adenylate cyclase assay the same assay conditions as for guanylate cyclase were employed, except that 1 mM [^{3}H]ATP (4 Ci/mole), 0.8 mM cyclic AMP, and 10 mM $MgCl_2$ were substituted for GTP, cyclic GMP, and 3 mM $MgCl_2$ respectively. After the assay mixture was incubated at 37° for 15 min, the reaction was terminated by heating for 2 min in a boiling bath, following the addition of 1 N HCl (40 μl). The radioactive cyclic GMP or cyclic AMP was isolated by the serial use of neutral aluminum oxide-Dowex 1-x2 column and the radioactivity was determined as described elsewhere [26]. Protein was determined by the method of Lowry *et al.* [27], with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Effect of phenothiazine derivatives on the activity of Tetrahymena *guanylate cyclase.* The procedure (see Materials and Methods) for preparing *Tetrahymena* particulate fraction yields a Ca^{2+}-dependent guanylate cyclase that can be maximally stimulated by 10 μg of TCBP to about 20-fold above the basal activity in the presence of 100 μM Ca^{2+}. TCBP does not influence the guanylate cyclase activity in the absence of Ca^{2+} [18]. On the other hand, 100 μM Ca^{2+} alone stimulated the guanylate cyclase activity

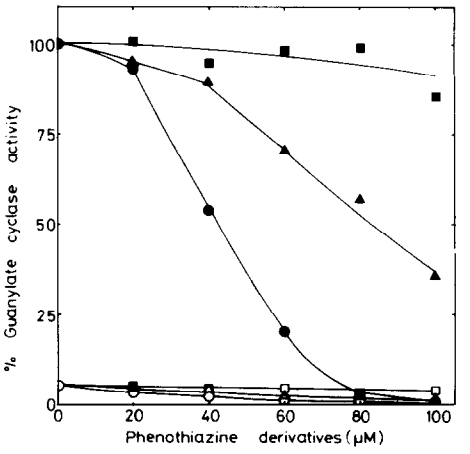

Fig. 1. Inhibition of *Tetrahymena* guanylate cyclase activity by phenothiazine derivatives in the presence and absence of TCBP. Guanylate cyclase activity of a preparation from *T. pyriformis* was measured in the absence of TCBP (open symbols) and in the presence of 10 μg TCBP (closed symbols) and various concentrations of phenothiazine derivatives: (○ – ●) trifluoperazine; (△ – ▲) chlorpromazine; and (□ – ■) propericiazine. Guanylate cyclase activity is related to TCBP-stimulated enzyme in the absence of the agents. The guanylate cyclase activity was 1100–1300 pmoles·(mg protein)$^{-1}$·min^{-1} in the presence of 100 μM Ca^{2+} and 10 μg TCBP.

without adding exogenous TCBP. This effect of Ca^{2+} on the enzyme activity may be explained by a minute amount of endogenous TCBP in the particulate fractions. The specific activity of the basal Ca^{2+}-dependent guanylate cyclase was 40–60 pmoles·(mg protein)$^{-1}$·min^{-1} and of the maximally activated 1100–1300 pmoles·(mg protein)$^{-1}$·min^{-1} respectively. Ca^{2+}-independent guanylate cyclase activity (in the presence of 0.5 mM EGTA) was less than 10 per cent of the total activity of the preparation. The effects of the phenothiazine derivatives—chlorpromazine, propericiazine, and trifluoperazine—on *Tetrahymena* Ca^{2+}-dependent guanylate cyclase are shown in Fig. 1. Chlorpromazine and trifluoperazine were potent inhibitors of both the basal Ca^{2+}-dependent guanylate cyclase activity (in the absence of TCBP) and the TCBP-activated fraction of the guanylate cyclase. Propericiazine showed only slight inhibitory action. The concentrations (IC_{50}) of trifluoperazine and chlorpromazine that produced 50 per cent inhibition of the basal Ca^{2+}-dependent guanylate cyclase activity were approximately 40 and 80 μM respectively. IC_{50} Values of the two drugs for the basal guanylate cyclase activity and the guanylate cyclase activation by TCBP were indistinguishable. On the other hand, the Ca^{2+}-independent guanylate cyclase activity was not influenced by the agents used (data not shown). This inhibition of the basal Ca^{2+}-dependent guanylate cyclase activity caused by the agents in the absence of TCBP might have been due either to the presence of membrane-bound TCBP or to incomplete removal of endogenous TCBP. In addition, we cannot exclude the alternative possibility that effects of the agents on the guanylate cyclase were due to their interactions with membranes rather than to their specific actions on TCBP, since these drugs contain hydrophobic moieties.

We also tested the effect of trifluoperazine or chlorpromazine on *Tetrahymena* adenylate cyclase activity, which was found to be present in a membrane-bound form and not to be influenced by TCBP [18]. In the concentration range examined (20–100 μM), these drugs had little or no effect on the adenylate cyclase activity (data not shown).

Effect of Ca^{2+} *on trifluoperazine-induced inhibition of guanylate cyclase activation.* Since the activation of guanylate cyclase requires Ca^{2+} [18], and trifluoperazine can complex Ca^{2+} [28], we determined whether increasing the concentration of Ca^{2+} could prevent the trifluoperazine-induced inhibition of guanylate cyclase activation. The results are shown in Fig. 2. When guanylate cyclase was measured in the presence of 10 μg TCBP without trifluoperazine, the enzyme activity responded to increasing Ca^{2+} concentrations biphasically, with about 20-fold maximal activation occurring at 50–100 μM added Ca^{2+}. We have presented evidence that the stimulatory action of Ca^{2+} is conferred on the enzyme activity by TCBP [18]. On the other hand, the inhibitory effects of Ca^{2+} on the guanylate cyclase activity may have resulted from competition for the sites at which Mg^{2+} enhances the reactivity of the catalytic site or from Ca^{2+} binding to distinct, allosteric inhibitory sites. The precise mechanism by which the enzyme activity is inhibited by higher concentrations of Ca^{2+} remains to be elucidated. In the presence of 10 μg TCBP, 40 μM trifluoperazine inhibited the activity of guanylate cyclase by approximately 50 per cent over the range of Ca^{2+} from 0 to 1.2 mM. This implies that Ca^{2+} concentration failed to prevent the trifluoperazine-induced inhibition of guanylate cyclase activation.

Effect of TCBP concentration on trifluoperazine-induced inhibition of guanylate cyclase activation. It was next determined whether the trifluoperazine-induced inhibition of guanylate cyclase activation could be overcome by increasing the concentration of TCBP. Figure 3 shows the effect of TCBP on

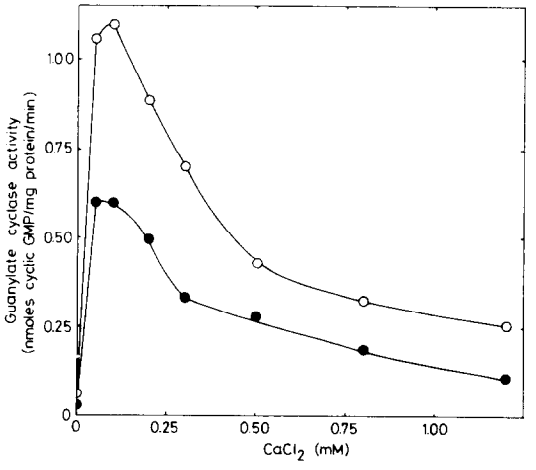

Fig. 2. Effect of increasing the $CaCl_2$ concentration on inhibition by trifluoperazine of guanylate cyclase activation. Guanylate cyclase activity of a preparation from *T. pyriformis* was measured in the absence of any inhibitors (○) and in the presence of 40 μM trifluoperazine (●) and various concentrations of $CaCl_2$. TCBP (10 μg) was present in all samples.

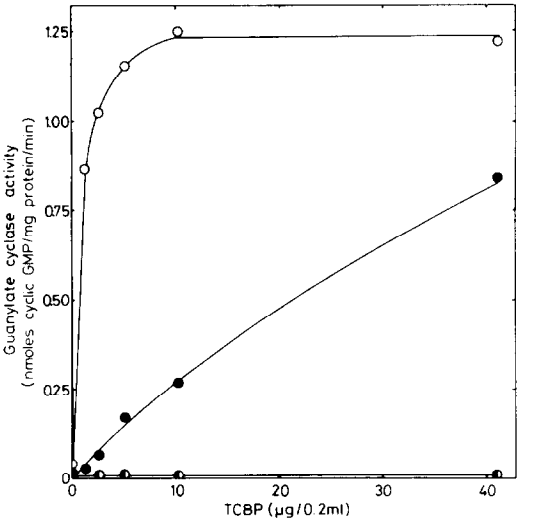

Fig. 3. Effect of TCBP concentration on inhibition by trifluoperazine or EGTA of guanylate cyclase activation. Guanylate cyclase activity of a preparation from *T. pyriformis* was measured in the absence of any inhibitors (○) and in the presence of 50 μM trifluoperazine (●) or 0.3 mM EGTA (◐) and various concentrations of TCBP.

guanylate cyclase activity in the presence and absence of trifluoperazine or EGTA. In the absence of inhibitors (control samples), increasing the concentration of TCBP produced a concentration-dependent increase in guanylate cyclase activity, which reached a maximum elevation of about 20-fold. In the absence of added TCBP, 0.3 mM EGTA produced about 90 per cent inhibition of guanylate cyclase activity. The endogenous TCBP in the enzyme preparation may account for the loss of the cyclase activity. The gradual increase of TCBP in the presence of EGTA failed to increase the activity of guanylate cyclase. In the absence of TCBP, 50 μM trifluoperazine inhibited guanylate cyclase activity to the same extent as did EGTA. Addition of increasingly higher concentrations of TCBP in the presence of trifluoperazine produced a progressive restoration of guanylate cyclase activity. At a TCBP concentration of 10 μg/sample, 50 μM trifluoperazine prevented activation by about 80 per cent, while at 40 μg TCBP sample 50 μM trifluoperazine caused about 35 per cent inhibition of the activation of guanylate cyclase. These results imply that the TCBP may competitively antagonize the trifluoperazine-induced inhibition of the activation of guanylate cyclase. To investigate further the interactions between TCBP, guanylate cyclase and trifluoperazine, the activity of guanylate cyclase was determined at various concentrations of TCBP and trifluoperazine (Fig. 4.). The data were plotted at $1/v$ vs 1/TCBP concentration. The point at which the lines cross the y axis represents the theoretical maximum velocity (V_{max}) obtained in the presence of TCBP. As can be seen, the V_{max}values for guanylate cyclase in the absence (control) and presence of 25 or 50 μM trifluoperazine were similar (approximately 1250 pmoles·(mg protein)$^{-1}$·min^{-1}). On the other hand, the apparent affinity of the TCBP for the guanylate cyclase decreased with increasing concentrations of

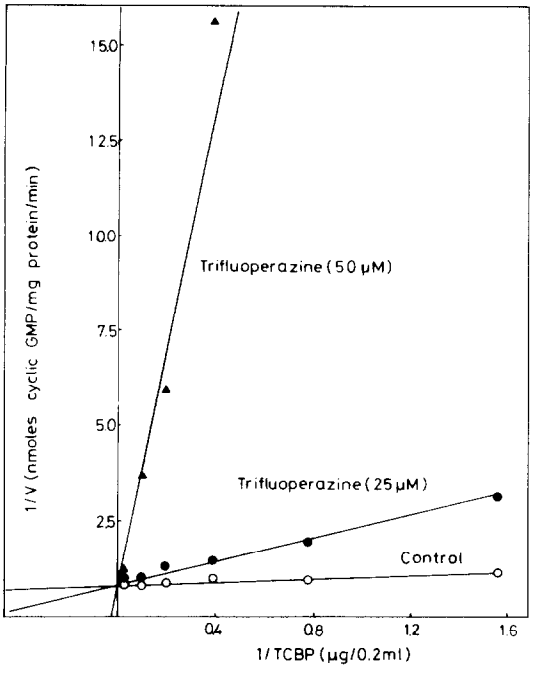

Fig. 4. Kinetic analysis of trifluoperazine-induced inhibition of activation of guanylate cyclase. The activity of *Tetrahymena* guanylate cyclase was measured in the presence of various concentrations of TCBP and trifluoperazine.

trifluoperazine. The apparent *Kd* values were 0.4 μg in the absence of trifluoperazine and 2.6 and 45μg in the presence of 25 and 50 μM trifluoperazine respectively. This is consistent with the idea that trifluoperazine is a competitive inhibitor of *Tetrahymena* guanylate cyclase activation, as observed with other calmodulin-dependent enzyme systems in various tissues [29]. It is currently believed that phenothiazine derivatives may prove to be a useful tool with which to elucidate Ca^{2+}- and calmodulin-dependent cell functions. These agents bind specifically to, and hence specifically block the various actions of, Ca^{2+}-calmodulin complexes. Therefore, the present data may show indirect evidence that the Ca^{2+}-dependent activator protein (TCBP) of *Tetrahymena* guanylate cyclase is identical to calmodulin. The data reported in this study indicate that trifluoperazine represses TCBP activation of guanylate cyclase activity to a greater extent than chlorpromazine does. In contrast, propericiazine showed little or no inhibitory effect. At the present moment, there is no clear explanation for such differences. Since a variety of hydrophobic ligands are known to interact nonspecifically with calmodulins [30], it is possible that the different activities may be related to the degree of hydrophobicity of these drugs. To date, the growth [31], motility [32], glucose utilization [31], and phosphate uptake [33] of *T. pyriformis* have been reported to be inhibited by low concentrations of chlorpromazine and phenothiazines. Although it seems premature to conclude that calmodulin plays a role in the above biological phenomena of this cell through the modulation of guanylate cyclase activity, further study on this protozoan system may provide a new clue to the role of cyclic GMP within the cell.

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