*Biochemical Pharmacology*, Vol. 32, No. 17, pp. 2501–2504, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00


# INHIBITORY EFFECTS OF CALMODULIN ANTAGONISTS ON PLASMA MEMBRANE CYCLASES IN *TETRAHYMENA*: CALMODULIN-DEPENDENT GUANYLATE CYCLASE AND CALMODULIN-INDEPENDENT ADENYLATE CYCLASE

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(*Received* 6 *January* 1983; *accepted* 10 *March* 1983)

**Abstract**—Trifluoperazine was shown previously to inhibit the activation of *Tetrahymena* guanylate cyclase activity by calmodulin [S. Nagao, S. Kudo and Y Nozawa, *Biochem. Pharmac.* **19**, 2709 (1981)]. The present paper reports that *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), another representative calmodulin inihibitor, inhibited the calmodulin-induced activation of the guanylate cyclase, and that trifluoperazine and W-7 also inhibited *Tetrahymena* adenylate cyclase. The adenylate cyclase activity was found to be present in a membrane-bound form and not to be influenced by calmodulin. The inhibitions of the adenylate cyclase activity by these agents were dose-dependent and not $Ca^{2+}$-dependent. These findings suggest that the inhibitory actions of these drugs may not necessarily be specific for calmodulin-dependent enzymes in *T. pyriformis*.

Calmodulin, an intracellular $Ca^{2+}$ receptor which is widely distributed in many cell types, is thought to play an important role in various aspects of cell regulation [1–4]. *In vitro* inhibitions of calmodulin-mediated responses are caused by various drugs: phenothiazines [5,6], butylophenones [7] and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) [8]. These agents are known to interact with calmodulin in a $Ca^{2+}$-dependent manner. Recent reports [9, 10] have indicated that a possible binding mechanism of these agents involves a hydrophobic region in calmodulin which is exposed by a $Ca^{2+}$-induced conformational change. Consequently, these calmodulin inhibitors are used to inhibit various cell functions in which calmodulin is involved [11–14], and also to isolate and purify calmodulins [15, 16].

In previous papers [17–20], we have demonstrated that a eukaryotic protozoan, *Tetrahymena pyriformis*, possesses a calmodulin which is unique in its capability to activate guanylate cyclase exclusively associated with the plasma membrane. This calmodulin was found to have the greatest variation in amino acid sequence among all calmodulins isolated so far [21]. Phenothiazines, such as trifluoperazine and chlorpromazine, interact with *Tetrahymena* calmodulin and, therefore, inhibit the calmodulin-induced activation of the guanylate cyclase activity [22]. However, since phenothiazines inhibit calmodulin through non-specific hydrophobic interactions, the antagonism of $Ca^{2+}$-dependent events by these agents was suggested to be an insufficient criterion for establishing a physiological role for calmodulin [23–25]. Recently, it was shown that several other cellular proteins, in addition to calmodulin, can interact in a $Ca^{2+}$-dependent manner with phenothiazine and W-7 [26].

Here we demonstrate that trifluoperazine and W-7 exert inhibitory effects on membrane-bound adenylate cyclase activity in *Tetrahymena*, which is unaffected by calmodulin, in a $Ca^{2+}$-independent manner.

## MATERIALS AND METHODS

*Materials*. [2-$^3$H]ATP and [8-$^3$H]GTP were purchased from the Radiochemical Centre (Amersham, U.K.). All unlabelled nucleotides used were obtained from Sigma Chemical Co. (St. Louis, MO). Creatine phosphate and creatine kinase were purchased from Boehringer Mannheim (F.R.G.). 3-Isobutyl-1-methylxanthine was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Neutral aluminium oxide was a product of Woelm Pharma (F.R.G.). Chlorpromazine, propericiazine and trifluoperazine were supplied by the Yoshitomi Pharmaceutical Co. (Osaka, Japan). *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was the generous gift of Dr. Hiroyoshi Hidaka, Mie University, Japan. Other chemical were obtained from commercial sources.

*Preparation of enzymes and calmodulin*. A thermotolerant strain of *T. pyriformis* (NT-1) was grown at 39.5° in an enriched proteose–peptone medium as described previously [27]. Cultures of 200 ml were harvested in the early stationary phase of growth. Plasma membrane, isolated using a phosphate buffer (0.2 M $KH_2PO_4$/3 mM EDTA/0.1 M NaCl, pH 7.2) according to the method of Nozawa and Thompson [27], was then washed twice with 10 mM Tris–HCl (pH 7.5), 250 mM sucrose, 1 mM dithiothreitol and 0.5 mM EGTA*, by homogenizing with this medium and then centrifuging. The final pellet was resus-

* Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; cyclic GMP, guanosine 3′,5′-monophosphate; cyclic AMP, adenosine 3′,5′-monophosphate.

pended and rehomogenized in the same Tris–HCl buffer without EGTA to give appropriate protein concentrations and was then used for enzyme assays. *Tetrahymena* calmodulin was purified as described previously [20,28].

*Adenylate and guanylate cyclase assay.* Unless otherwise indicated, the standard assay mixture for adenylate cyclase contained 1 mM [$^3$H]ATP (4 Ci/mole), 0.8 mM cyclic AMP, 15 mM creatine phosphate, 20 $\mu$g creatine kinase, 1 mM dithiothreitol, 1·5 mM isobutylmethylxanthine, 10 mM $MgCl_2$, 100 $\mu$M $CaCl_2$, 25 mM Tris–maleate (pH 6.8) and 100 $\mu$g of enzyme protein in a total volume of 0.2 ml. For the guanylate cyclase assay the same assay conditions for adenylate cyclase were employed, except that 1 mM [$^3$H]GTP (5 Ci/mole), 1 mM cyclic GMP and 3 mM $MgCl_2$ were substituted for ATP, cyclic AMP and 10 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 $\mu$l). The radioactive cyclic AMP or cyclic GMP was isolated by the serial use of neutral aluminium oxide-Dowex 1-X2 column and the radioactivity was determined as described elsewhere [29].

Protein was determined by the method of Lowry *et al.* [30], with bovine serum albumin as standard.

## RESULTS AND DISCUSSION

In previous papers [19, 31], we showed that both adenylate and guanylate cyclase activities in *T. pyriformis* are entirely localized in plasma membrane, but are not found in the soluble fraction. The procedure (see Materials and Methods) for preparing the plasma membrane yields a calmodulin-independent adenylate cyclase and a calmodulin-dependent guanylate cyclase. The latter can be maximally stimulated by 5 $\mu$g of *Tetrahymena* calmodulin to an approximately 10-fold increase above its basal activity in the presence of 100 $\mu$M $Ca^{2+}$. *Tetrahymena* calmodulin does not influence the guanylate cyclase activity in the absence of $Ca^{2+}$ [18]. Moreover, $Ca^{2+}$ alone does not stimulate the cyclase activity. This insensitivity of the enzyme activity to $Ca^{2+}$ makes it a more useful system for investigating effects of calmodulin inhibitors. The effects of trifluoperazine, chlorpromazine, propericiazine and W-7 on guanylate cyclase activity were examined in the presence and absence of *Tetrahymena* calmodulin (Fig. 1). Trifluoperazine, W-7 and chlorpromazine produced a concentration-dependent inhibition of the calmodulin-mediated stimulation of guanylate cyclase, and propericiazine exerted a small inhibitory action. However, the basal guanylate cyclase activity (in the absence of calmodulin) was not influenced by these agents. Since the activation of guanylate cyclase activity by calmodulin requires $Ca^{2+}$ [18], it is possible that the inhibition observed in Fig. 1 may result from chelating $Ca^{2+}$ with these agents. In order to examine whether $Ca^{2+}$ or calmodulin would play a primary role in the enzyme activity inhibition by these agents, the assay was established for a representative drug with a low $K_i$ (i.e. trifluoperazine). Trifluoperazine-induced repression of guanylate cyclase activation could be reversed by the addition

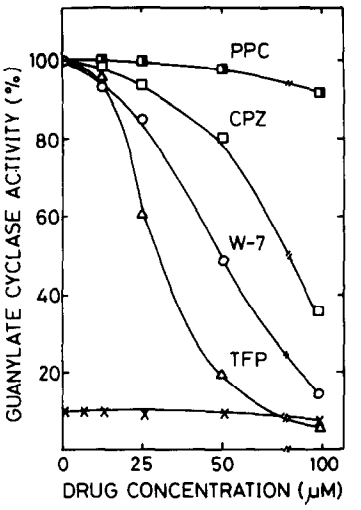


Fig. 1. Effects of phenothiazines and W-7 on guanylate cyclase activity of *Tetrahymena* plasma membrane in the presence and absence of calmodulin. Guanylate cyclase of *Tetrahymena* plasma membrane was measured in the absence of calmodulin and various concentrations of trifluoperazine (×), and in the presence of 5 $\mu$g calmodulin and various concentrations of drugs: trifluoperazine (△); W-7 (○); chlorpromazine (□); and propericiazine (◨). The results are expressed as percentages of maximal activity in the presence of 5 $\mu$g of calmodulin and 0.1 mM $Ca^{2+}$. The maximal activity is 1260 pmole/min per mg protein.

of calmodulin but not $Ca^{2+}$ (data not shown). These results provide support for the concept that the agent acts by binding to calmodulin and thereby inhibits the guanylate cyclase activity, as observed with cyclic nucleotide phosphodiesterase [5], adenylate cyclase [6] and $Ca^{2+}$–$Mg^{2+}$ ATPase [32] in other tissues. The concentrations of agents producing 50% inhibition ($IC_{50}$) of the guanylate cyclase activation by calmodulin are shown in Table 1. Trifluoperazine represses calmodulin-mediated activation of the cyclase activity to a greater extent than W-7 or chlorpromazine. At present, there is no clear explanation for differences among drugs in the inhibitory potency. However, since these agents have been shown to inhibit calmodulin's functions through hydrophobic interaction [9,10], it is possible that their different activities may be related to the degree of hydrophobicity of drugs.

The effects of trifluoperazine and W-7 on *Tetrahymena* adenylate cyclase activity were examined in the presence and absence of $Ca^{2+}$ (Fig. 2). As previously reported [18], the adenylate cyclase activity was not activated by calmodulin with or without the addition of $Ca^{2+}$. Rather $Ca^{2+}$ alone at 100 $\mu$M was found to decrease the enzyme activity. It should be noted that both trifluoperazine and W-7 showed marked inhibition of the adenylate cyclase activity in a concentration-dependent manner.

The $IC_{50}$ values of the enzyme activity are shown in Table 1. The $IC_{50}$ values for W-7 in the presence and absence of $Ca^{2+}$ was lower than that for trifluoperazine. The $IC_{50}$ value for W-7 plus $Ca^{2+}$ was lower than that for W-7 minus $Ca^{2+}$, while the $IC_{50}$ value for trifluoperazine plus $Ca^{2+}$ was higher than that for trifluoperazine minus $Ca^{2+}$.

Table 1. Effects of phenothiazines and W-7 on calmodulin-induced activation of guanylate cyclase and adenylate cyclase in *Tetrahymena* plasma membrane

| | $IC_{50}$ (μM)* | | |
|---|---|---|---|
| | Guanylate cyclase | Adenylate cyclase | |
| Drugs | 100 μM $Ca^{2+}$ + 5 μg of calmodulin | 100 μM $Ca^{2+}$ | 1 mM EGTA |
| Trifluoperazine | 28 | 128 | 100 |
| W-7 | 46 | 45 | 75 |
| Chlorpromazine | 85 | n.d.† | n.d. |
| Propericiazine | 100 < | n.d. | n.d. |

* The concentration of drug that produced 50% inhibition of enzyme activity of *Tetrahymena* plasma membrane. Each value is the mean of at least two determinations.
† Not determined.

These results demonstrate that the calmodulin antagonists W-7 and phenothiazine inhibit the activation induced by the $Ca^{2+}$-calmodulin complex of the guanylate cyclase in *T. pyriformis*. It thus appears that the inhibition of the guanylate cyclase was thought to occur by interfering with the interaction of the enzyme with the $Ca^{2+}$-calmodulin complex. The inhibitory effects may not necessarily be specific for calmodulin. However, since these antagonists also inhibit the adenylate cyclase activity, which is calmodulin-independent in this organism, these hydrophobic agents may cause membrane alterations that are independent of calmodulin [25,33]. Although the precise mechanism by which the adenylate cyclase was inhibited in the presence of calmodulin antagonists remains to be elucidated, it is possible that these agents interact non-specifically with membranes, probably the lipid bilayer, thus eliciting an inhibition of the adenylate cyclase activity. Our preliminary observations by ESR analysis indicate that the physical state of liposomes was affected by the drugs (unpublished data). Consequently, when these agents are applied to biological systems, care should be taken to interpret the observed findings.

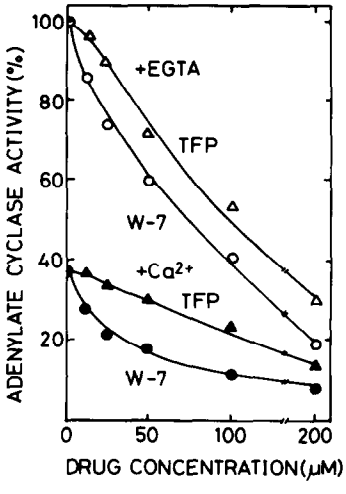


Fig. 2. Effects of trifluoperazine and W-7 on adenylate cyclase activity of *Tetrahymena* plasma membrane. Adenylate cyclase activity of *Tetrahymena* plasma membrane was measured in the presence of 100 μM $Ca^{2+}$ (▲, ●) or 1 mM EGTA (△, ○), and various concentrations of trifluoperazine (△, ▲) and W-7 (○, ●). Values for the coordinate were expressed as percentages of the maximal adenylate cyclase activity and were corrected for the enzyme activity in the presence of 1 mM EGTA without addition of the agents. The maximal enzyme activity was 140 pmole/min per mg protein.

*Acknowledgements*—We wish to thank Dr. Hiroyoshi Hidaka of the Department of Pharmacology, University of Mie, for generously providing W-7. This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan.

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