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INHIBITION BY PHENOTHIAZINE DERIVATIVES OF THE ADENYLATE CYCLASE OF AMPHIBIAN OOCYTES

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Abstract—The adenylate cyclase activity of membranes of *Xenopus laevis* oocytes and follicle cells was affected by the presence of 2-chloro-10-(3-aminopropyl)phenothiazine (CAPP) and two other antipsychotic drugs, fluphenazine and penfluridol. CAPP, at concentrations of 10 and 100 μM, had opposite effects on the activation of the oocyte adenylate cyclase by effectors that act through the G/F regulatory subunit. Under these conditions, the drug stimulated the activation by fluoride and drastically inhibited the activation by guanyl-5′-yl-imidodiphosphate [Gpp(NH)p] and by cholera toxin and GTP. The activity of the catalytic subunit measured in the presence of either Mn^{2+} or forskolin was not affected by 100 μM CAPP. however, concentrations of this drug above 100 μM inhibited the adenylate cyclase activated by fluoride or by forskolin and also inhibited the activity of a calmodulin-independent cyclic nucleotide phosphodiesterase present in the same oocyte membrane preparation. Oocyte adenylate cyclase has been shown previously to be inhibited by the hormone progesterone. The inhibitory effect of CAPP is additive to that measured with the hormone, indicating that these compounds act through different mechanisms. CAPP did not modify the concentration of Gpp(NH)p required to yield half-maximal activation and, although the drug inhibited more strongly at lower concentrations of Gpp(NH)p, saturating amounts of the guanine nucleotide did not reverse completely the inhibition caused by CAPP. The effects of these antipsychotic drugs on oocyte adenylate cyclase did not require the presence of free Ca^{2+} and were not altered by the addition of exogenous calmodulin and calcium.

Several reports have demonstrated that phenothiazine derivatives, well-known antipsychotic drugs, can affect the meiotic maturation of amphibian oocytes induced by progesterone [1–3]. A report by Cartaud *et al.* [3] indicates that concentrations of fluphenazine below 200 μM can enhance the hormonal induction of maturation, while higher concentrations of the drug inhibit this process. Since these drugs can bind calmodulin in the presence of calcium and are known to inhibit enzymes activated by this protein modulator [4], the previous findings have been interpreted to support the hypothesis that calmodulin is involved in the hormonal induction of oocyte maturation.

Since the induction of maturation by progesterone is accompanied by a transient decrease of the cAMP concentration of the oocyte [5–7], our recent research about this process has concentrated on the enzymes that regulate the levels of cAMP in the cell: adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] and cyclic nucleotide phosphodiesterase [3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17].

It has been found that *Xenopus laevis* oocytes contain a species of cyclic nucleotide phosphodiesterase that can be greatly stimulated by calmodulin and Ca^{2+} and which is inhibited by 2-chloro-10-(3-aminopropyl)phenothiazine (CAPP)† [8]. This enzyme is inhibited in the intact cell [9] and, as yet, no direct evidence has related it to the maturation process.

In this same species, oocyte adenylate cyclase has been found to be regulated by a G/F subunit as shown by its activation by hydrolysis-resistant guanine nucleotides, fluoride ions and cholera toxin [10]. Progesterone inhibits *in vitro* and *in vivo* oocyte adenylate cyclase with characteristics of concentration and specificity that strongly support the idea that this novel effect of a steroid hormone is related to the induction of the meiotic maturation of these cells [11–15].

The data of this report demonstrate that CAPP and two other antipsychotic drugs were potent inhibitors of the adenylate cyclase of *X. laevis* oocytes and follicle cells. It is further shown that the G/F subunit of the adenylate cyclase was sensitive to CAPP at concentrations below 100 μM while higher concentrations of the drug affected non-specifically the catalytic subunit of the enzyme and also membrane-bound cyclic nucleotide phosphodiesterase. The effects of the phenothiazine derivatives on this adenylate cyclase seemed to be independent of calmodulin because they did not require the presence of free Ca^{2+} and were not modified by the addition of exogenous calmodulin and Ca^{2+}.

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† Abbreviations: CAPP, 2-chloro-10-(3-aminopropyl)-phenothiazine; fluphenazine, 2-(trifluoro-methyl)-10-[3-{1(β-hydroxyethyl)-4-piperazinyl}propyl]phenothiazine; penfluridol, 1-[4,4-bis(4-fluorophenyl)butyl]-4-hydroxy-4-(3-trifluoromethyl-4-chlorophenyl)piperidine; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethylether)*N,N*-tetracetic acid; CaM, calmodulin; and Hepes, 4-(2-hydroxyethyl)-1-pieperazine-ethanesulfonic acid.

MATERIALS AND METHODS

Isolation of oocytes and follicle cells. Adult female *X. laevis* were obtained from the South African Snake Farm, Cape Province, R.S.A. Animals were anesthetized in ice water, and the ovaries were removed, cut into small pieces, and incubated with 0.2% collagenase for 3 hr at 22° to defolliculate the oocytes. Stage VI oocytes were selected by sieving through a 700 μm Nitex screen, and subsequently the cells retained were separated under the microscope to isolate oocytes that were 1.2 mm in diameter.

Follicle cells were obtained by passing the cell suspension resulting after the collagenase treatment of the ovary through Nitex screens of 700 and 500 μm pore size, and finally the liquid was passed through a fine screen of 53 μm pore size. The cells retained in the last sieving were essentially free of oocytes and were resuspended and centrifuged for 10 min at 1000 *g*. The sediment contained the follicle cells which were examined under the microscope to remove contaminants manually.

Preparation of oocyte and follicle cell homogenates and particulate adenylate cyclase. Defolliculated oocytes were homogenized in 1 vol. of a solution containing 0.88 M sucrose, 50 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol and 1 mM EGTA. A Dounce homogenizer was used for grinding the cells with ten strokes of pestle A and ten strokes of pestle B. The homogenate was centrifuged at 1000 *g* and 20,000 *g*, and finally a 105,000 *g* particulate fraction was prepared as described previously [14]. In most of the experiments, the particulate fraction was resuspended in 50 mM Tris–HCl (pH 7.4) with 1 mM dithiothreitol and pelleted twice in the same buffer to eliminate the EGTA used in the homogenization.

Follicle cells obtained after collagenase treatment of the ovary were homogenized in 10 vol. of 0.44 M sucrose, 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol and 1 mM EGTA. The fraction sedimenting at 20,000 *g* contained 80% of the adenylate cyclase activity and was used as a source of the enzyme.

Assay for adenylate cyclase. Adenylate cyclase activity of the oocyte and follicle cell membrane fraction was assayed by the procedure of Rodbell [16]. Incubations were carried out at 32° for 20 min in 100 μl containing: 50 mM Tris–HCl (pH 7.4), 1 mM cAMP, 2 mM 3-isobutyl-1-methylxanthine, 1 mM dithiothreitol, 0.3 mM EDTA, 5% glycerol, 5 mM $MgCl_2$, 4 mM creatine phosphate, 0.4 mg/ml creatine kinase, 100–120 μM [α^{32}P]ATP (300–1200 cpm/pmole) and 50–300 μg of protein of the membrane fractions. Incubations were stopped by the addition of 0.1 ml of 15 mM ATP and 3 mM [^{3}H]cAMP (4000 cpm/pmole) and heating for 3 min at 100°. [^{32}P]cAMP formed was analyzed as described by Salomon *et al.* [17].

Preparation of calmodulin-dependent cyclic nucleotide phosphodiesterase from oocytes. The preparation of oocyte cyclic nucleotide phosphodiesterase that can be greatly activated by calmodulin and Ca^{2+} was essentially as previously described [8]. This enzyme preparation had a basal activity of 35 pmoles cAMP hydrolyzed per mg protein per min and in the presence of calmodulin and Ca^{2+} the cAMP hydrolysis reached 300 pmoles per mg protein per min.

Assay for cyclic nucleotide phosphodiesterase. The procedure of Thompson *et al.* [18] was used. The standard incubation contained in 200 μl: 10 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 3 mM β-mercaptoethanol, 0.1 mM EDTA, 0.1 mM EGTA, and 1 to 5×10^5 cpm of [^{3}H]cAMP with a 5 μM concentration of this substrate.

After 20 min at 30°, the reaction was stopped by boiling for 90 sec, and 25 μl of snake venom 5-nucleotidase (2 mg/ml) was added to the cooled tubes. A second incubation was for 10 min at 30° and was stopped by adding 1 ml ethanol. Aliquots of 0.5 ml were applied to 0.3 ml Dowex-1-X4,Cl (200–400 mesh) columns. These columns were eluted with 1.5 ml methanol, collecting the eluant directly in scintillation vials for counting.

Treatment of membranes with cholera toxin. The membranes that contained the adenylate cyclase activity were treated with cholera toxin exactly as described previously [10].

Sources of materials. CAPP was obtained from the Psychopharmacology Research Branch, National Institute of Mental Health, N.I.H., Bethesda, Md. Fluphenazine was obtained from the Schering Co. and penfluridol from Jansen Chemikalien.

[α^{32}P]ATP was prepared as described by Walseth and Johnson [19] using [^{32}P]phosphate from ICN. [^{3}H]cAMP was from New England Nuclear.

Progesterone and forskolin were from Calbiochem. Cholera toxin was obtained from Schwarz/Mann. Gpp(NH)p, ATP, cAMP and creatine phosphate were from the Sigma Chemical Co. 3-Isobutyl-1-methylxanthine was purchased from Aldrich and creatin kinase from Boehringer. Bovine brain calmodulin was a gift of Dr. C. Klee.

RESULTS

The results of experiment 1 in Table 1 demonstrate that 100 μM CAPP and two other antipsychotic drugs were potent inhibitors of the oocyte adenylate cyclase measured in the presence of Gpp(NH)p. The inhibition by the drugs was greater than that observed with progesterone. Previous work [14] has shown that 1 μM progesterone yields maximal inhibition by this hormone and that this varies from 30 to 60%, with different adenylate cyclase preparations. The assays of this experiment were carried out in the presence of 0.5 mM EGTA, and the membranes used as source of enzyme were also prepared in the continued presence of a 1 mM concentration of this Ca^{2+} chelator. It is clear, therefore, that the inhibition by antipsychotic drugs and by progesterone does not require free calcium ions. In experiment 2, EGTA was not added to the assay mixture and the membranes were washed in a buffer that contained neither EGTA nor Ca^{2+}. Addition of Ca^{2+} and calmodulin resulted in a small but significant inhibition of the oocyte adenylate cyclase. This inhibition was also observed when Ca^{2+} was added in the absence of calmodulin, a finding that has been reported previously [13]. The addition of EGTA did not affect the enzyme activity. Experiment 3, using similar conditions as experiment 2, demonstrates

Table 1. Effects of antipsychotic drugs, Ca^{2+} and calmodulin on the oocyte adenylate cyclase*

Expt.	Additions	Adenylate cyclase activity (pmoles cAMP/mg protein)	Inhibition (%)
1	None	151 ± 4	
	CAPP (100 μM)	58 ± 1	62
	Fluphenazine (100 μM)	75 ± 2	50
	Penfluridol (100 μM)	48 ± 1	68
	Progesterone (1 μM)	101 ± 10	33
2	None	100 ± 1.5	
	CaM (50 μM) and Ca^{2+} (50 μM)	77 ± 2	23
	EGTA (500 μM)	106 ± 2	
3	None	78 ± 1.6	
	CAPP (100 μM)	26 ± 0.3	66
	CAPP (100 μM) + CaM (50 μM) + Ca^{2+} (50 μM)	30 ± 1.4	61

* The preparation of the enzyme and its assay was as described in Materials and Methods except that in experiment 1 the membranes containing the enzyme were not washed free of EGTA by resedimentation and, in addition, the assay mixture contained: 0.5 mM EGTA, 25 μM Gpp(NH)p, 60 μg of enzyme protein and 0.1 mM [α^{32}P]ATP (445 cpm/pmole). In experiments 2 and 3, the membrane-bound enzyme was washed free of EGTA by two sedimentations as described in Materials and Methods. In these assays no EGTA was added except where specified and 50 μg of enzyme protein and 0.1 mM [α^{32}P]ATP (964 cpm/pmole) were used. The assays of the three experiments were incubated for 20 min at 32° and the ± indicate the standard deviation of the triplicate results.

that the inhibition by CAPP was not affected by the addition of exogenous calcium and calmodulin.

The effect of different concentrations of CAPP on the activity of adenylate cyclase and cyclic nucleotide phosphodiesterase is presented in Fig. 1. Part A shows the effect of drug on the adenylate cyclase activated by two effectors of the G/F subunit, Gpp(NH)p and fluoride ions. The activity obtained with F^- indicates that 10–100 μM concentrations of CAPP stimulated the adenylate cyclase while concentrations above 100 μM caused a strong inhibition of the enzyme. The same concentrations that activated the enzyme with F^- caused a drastic inhibition of the enzyme measured in the presence of Gpp(NH)p.

Figure 1B shows a similar experiment assayed with the enzyme in the presence of forskolin, a known activator of the catalytic subunit of the adenylate cyclase of many tissues [20]. Forskolin activated the oocyte enzyme up to 6-fold at a concentration of 100 μM. The adenylate cyclase activity was not altered by the addition of up to 100 μM CAPP, yet above this level it was strongly inhibited.

Figure 1C presents the effect of the same drug on two different cyclic nucleotide phosphodiesterases present in *X. laevis* oocytes, one a soluble calmodulin/Ca^{2+} stimulated enzyme and the other a membrane bound, low K_m enzyme. With the cytosol enzyme, the stimulation caused by calmodulin and Ca^{2+} was inhibited by CAPP concentrations between 10 and 50 μM, as had been shown previously [8]. On the other hand, the inhibition of membrane bound phosphodiesterase activity required concentrations of the drug above 100 μM. This membrane enzyme which was not activated by calmodulin and calcium nor by cGMP (unpublished results) is a constituent of the membranes used routinely for the adenylate cyclase activity measurements.

CAPP at a 100 μM concentration strongly inhibited the oocyte adenylate cyclase activated by cholera toxin and GTP but did not inhibit the basal activity or the activity of the enzyme stimulated by the substitution of Mn^{2+} for Mg^{2+} (Table 2). It is known that cholera toxin acts through the G/F subunit [21] while Mn^{2+} stimulates the catalytic subunit of adenylate cyclase. In the presence of Gpp(NH)p, several concentrations of CAPP also inhibited the adenylate cyclase present in the follicle cells that surround the oocytes in the *X. laevis* ovary (Table 2). The enzyme activity measured with these cells is not inhibited by progesterone [15].

Figure 2 shows the effect of different CAPP concentrations on the activity of adenylate cyclase assayed in the presence and absence of 10 μM progesterone. The results obtained indicate that the inhibition caused by progesterone, approximately 40%, was additive to that caused by the drug throughout the different concentrations of CAPP.

The inhibitory capacity of 100 μM CAPP was measured as a function of different concentrations of Gpp(NH)p (Fig. 3). From the results obtained, it is evident that the drug did not modify significantly the Gpp(NH)p concentration required to give half-maximal activation of the enzyme, which in both cases was approximately 5 μM. It is also noteworthy that, at 1 μM Gpp(NH)p, CAPP completely inhibited the activation of the enzyme by the guanine nucleotide. However, saturating concentrations of the guanine nucleotide did not reverse the inhibition by CAPP, which remained at about 50%.

DISCUSSION

The results presented above demonstrate that CAPP and two other antipsychotic drugs affected the activity of the adenylate cyclase present in the membrane fraction of *X. laevis* oocytes.

Thirteen years ago, Wolff and Jones [22] reported

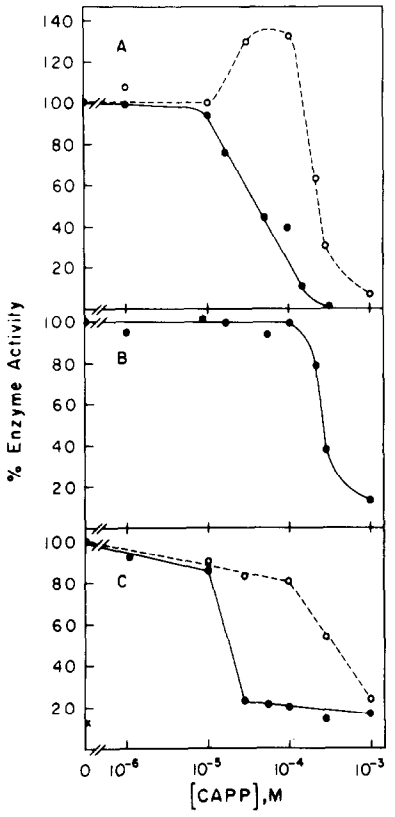

Fig. 1. Effect of CAPP concentrations on the activities of adenylate cyclase and cyclic nucleotide phosphodiesterase from *X. laevis* oocytes. In panels A and B, membrane-bound adenylate cyclase was prepared from oocytes and assayed in triplicate as described in Materials and Methods. In (A), in addition to the standard reagents, the assay contained either 5 mM NaF, 50 μg of enzyme protein, 0.2 mM EGTA and 0.1 mM [α^{32}P]ATP (832 cpm/pmole) with 100% of the activity corresponding to 151 pmoles of cAMP formed/mg protein in a 20 min incubation (O---O); or 25 μM Gpp(NH)p, 60 μg of enzyme protein, 0.5 mM EGTA and 0.1 mM [α^{32}P]ATP (444 cpm/pmole) with 100% activity corresponding to 151 pmoles of cAMP formed/mg protein in 20 min (●—●). In (B), the assay contained, in addition, 100 μM forskolin, 100 μg of enzyme protein, 0.2 mM EGTA, and 0.1 mM [α^{32}P]ATP (220 cpm/pmole) with 100% activity corresponding to 80 pmoles of cAMP formed/mg protein in 20 min. In (C), the cyclic nucleotide phosphodiesterase activity of a calmodulin-dependent enzyme or the activity present in the membrane fraction prepared as a source of adenylate cyclase was assayed as described in Materials and Methods. The oocyte calmodulin-dependent enzyme (●—●) was partially purified as described previously [8], and the assay contained in addition: 0.5 mM Ca^{2+}, 5 μM bovine brain calmodulin, and 20 μg of enzyme protein. In this case, 100% activity corresponded to 301 pmoles of [^{3}H]cAMP hydrolyzed/mg protein per min (1 pmole of [^{3}H]cAMP = 370 cpm). The symbol (X) denotes the activity of this enzyme in the absence of calmodulin and Ca^{2+} and in the absence of CAPP. The calmodulin-independent cAMP phosphodiesterase present in the membrane fractions (O---O) was assayed in the presence of 0.25 mM EGTA using 110 μg of enzyme protein. One hundred percent activity corresponded to 30 pmoles of cAMP hydrolyzed/mg protein per min.

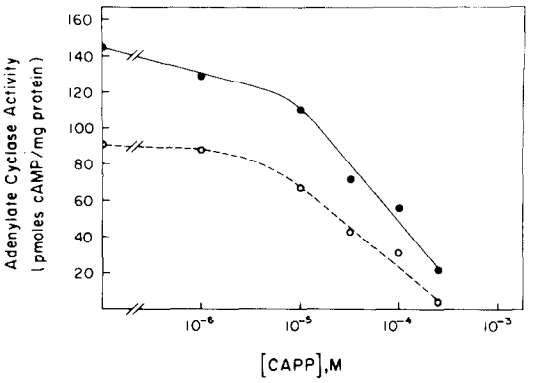

Fig. 2. Effect of progesterone on the inhibition of adenylate cyclase by CAPP. The oocyte adenylate cyclase preparation was assayed as described in Materials and Methods and included 0.2 mM EGTA, 0.1 mM [α^{32}P]ATP (944 cpm/pmole) and 50 μg of enzyme. The assay mixture was incubated for 20 min at 32° and was carried out in the presence (O---O) or absence (●—●) of 10 μM progesterone.

that phenothiazines inhibit the hormonal activation of adenylate cyclase of some mammalian tissues but have no effect on the enzyme of other tissues. These authors also showed that the adenylate cyclases which are inhibited by these drugs when measured in the presence of hormones are stimulated when assayed with fluoride ions.

In the light of our present knowledge about the regulation of adenylate cyclase and about some of the biochemical interactions of these antipsychotic drugs, we can attempt to interpret the earlier results of Wolff and Jones [22] and those presented above. It seems unlikely that the effects of the phenothiazines on oocyte adenylate cyclase were mediated through calmodulin since free Ca^{2+} was not required and addition of exogenous calmodulin and Ca^{2+} did not modify the effect of the drug. Rather, it seems more probable that these effects were related to the

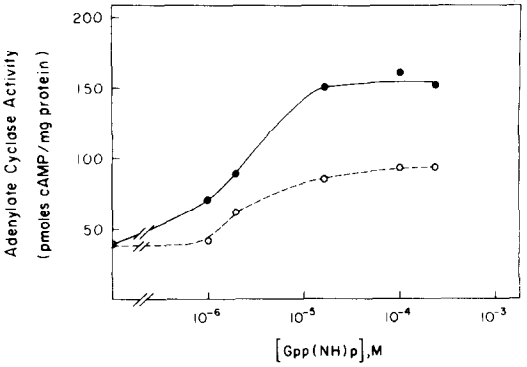

Fig. 3. Effect of CAPP on the activation of oocyte adenylate cyclase by Gpp(NH)p. The oocyte adenylate cyclase was prepared and assayed as described in Materials and Methods. The assay mixture was incubated for 20 min at 32°. The assays contained the materials specified in Materials and Methods, including 100 μg of enzyme protein, 0.1 mM [α^{32}P]ATP (101 cpm/pmole), 0.2 mM EGTA, and the amounts of Gpp(NH)p indicated and were carried out in the presence (O---O) and absence (●—●) of 100 μM CAPP.

Table 2. Effect of CAPP on the activity of adenylate cyclase of oocytes and follicle cells*

Source of enzyme	Additions	Activity (pmoles cAMP/mg protein) Without CAPP	With 100 μM CAPP	% Inhibition
Oocytes	None	35.0 ± 4	36.0 ± 4	0
	Gpp(NH)p (25 μM)	151 ± 4	58 ± 1	61
	Cholera toxin† and GTP (25 μM)	94 ± 4	46 ± 1	51
	$MnCl_2$‡ (5 mM)	17.4 ± 0.5	17.4 ± 1	0
Follicle cells	Gpp(NH)p (25 μM)	418 ± 6	195 ± 2	53

* The preparation of the membranes containing the enzymes from both cell types and the general assay are described in Materials and Methods. For the oocyte enzyme, 60 μg of enzyme protein was used per assay, and the incubation was carried out in the presence of 0.5 mM EGTA. The substrate was 0.1 mM [α^{32}P]ATP (641 cpm/pmole). For the follicle cell enzyme, 10 μg of enzyme protein was used and the assay contained 0.2 mM EGTA. The substrate was 0.1 mM [α^{32}P]ATP (1790 cpm/pmole).

† In this experiment, the membrane-bound enzyme was pretreated with cholera toxin as described previously [10], and the incubation was carried out in the presence of 25 μM GTP.

‡ In this experiment the assay mixture did not contain 5 mM $MgCl_2$ which was replaced by 5 mM $MnCl_2$.

action of phenothiazines on membrane structure or organization due to non-specific hydrophobic interactions of these drugs [23]. It has been demonstrated recently that phenothiazines at concentrations similar to those required for the effects on adenylate cyclase cause stabilization of the membranes of red blood cells [24] and that some of their pharmacological effects are not related to their capacity to bind calmodulin [25]. Our data, therefore, do not support the proposals that relate the effect of phenothiazines on oocyte meiotic maturation to the possible activation of the adenylate cyclase of these cells by calmodulin [2, 3].

The G/F regulatory subunit, which is membrane bound as is the catalytic subunit, seems to be especially sensitive to the effects of phenothiazines. Concentrations of CAPP between 10 and 100 μM altered the activity stimulated by effectors that act through the G/F subunit such as hormones, guanine nucleotides and fluoride ions. On the other hand, these concentrations of the drug did not affect the activity of the catalytic subunit measured with forskolin or with Mn^{2+}.

Concentrations below 100 μM CAPP stimulated the activation by F^- and inhibited the activation of G/F dependent on guanine nucleotides as measured in the presence of Gpp(NH)p, cholera toxin and GTP, and hormones, as reported by Wolff and Jones [22]. This opposing response to CAPP suggests that guanine nucleotides and fluoride have different effects on the G/F subunit and on its interaction with the catalytic subunit.

Concentrations of CAPP above 100 μM apparently affected the membrane in a general fashion and caused the inhibition of the adenylate cyclase stimulated either fluoride or forskolin and the inhibition of the calmodulin-independent cyclic nucleotide phosphodiesterase that is bound to the same membranes. Wolff and Jones [22] also observed partial inhibition of the (Na^+, K^+)-ATPase of thyroid membranes at 120 μM chlorpromazine which is considerably higher than the concentration of the drug required to inhibit completely the stimulation of adenylate cyclase by thyrotropin in these membranes.

The inhibition by 100 μM CAPP of oocyte adenylate cyclase is similar to the inhibition of this enzyme caused by 1 μM progesterone in its requirement for guanine nucleotides [14]. However, it is clear that the two inhibitors do not share similar mechanisms, since their effects were additive at many different CAPP concentrations. Also, the inhibition by CAPP affects the adenylate cyclase of follicle cells which is not responsive to progesterone [15]. Another difference is that progesterone does not cause a stimulation of the oocyte enzyme in the presence of F^-.

The additive nature of the inhibition of a phenothiazine derivative and progesterone could explain why fluphenazine concentrations below 200 μM are found to potentiate the action of the hormone on the oocyte [3]. The inhibition of maturation observed at higher concentrations of the drug would be the result of the general effect of these derivatives on the oocyte membrane structure. Phenothiazine derivatives have been shown to induce parthenogenetic activation of mouse oocytes [26]. These observations may be related to the activity of these drugs on the adenylate cyclase of amphibian oocytes described here.

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