Progesterone Inhibits Adenylate Cyclase In Xenopus Oocytes

ACTION ON THE GUANINE NUCLEOTIDE REGULATORY PROTEIN

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Previous studies have shown that ripe Xenopus oocytes, 1.4 mm in diameter and suitable for microinjection, undergo meiotic cell division in response to progesterone by a mechanism involving a decrease in the level of cAMP. In order to investigate the mechanism by which the level of cAMP is reduced by progesterone treatment, adenylate cyclase activity was measured in manually dissected plasma membranes of oocytes in the presence of agents known to activate adenylate cyclase in other systems. Manually dissected oocyte plasma membranes express a basal adenylate cyclase activity of 1-5 pmol/mg/h, and this activity is reduced by 50% in the presence of progesterone. The oocyte adenylate cyclase is activated by pretreatment of intact oocytes with cholera toxin as well as by microinjection of the A subunit of toxin into the cell. Activation by holotoxin is concentration dependent with half-maximal stimulation at a concentration of 1 pm toxin, and the time course of toxin activation is sigmoidal with a lag of approximately 30 min before significant elevation of activity. Up to 60% of cholera toxin activation of adenylate cyclase is prevented by progesterone in a dose-dependent manner, with half-maximal inhibition at 1 µm progesterone, and this effect is not observed with steroids unable to induce meiotic cell division such as 17β-estradiol.

The activation of adenylate cyclase by microinjected A subunit is prevented totally by progesterone, suggesting that progesterone effects some step of toxin action subsequent to binding and A subunit internalization. Sodium fluoride (F⁻), guanylyl-5'-yl imidodiphosphate (Gpp(NH)p), and manganese each stimulate the oocyte adenylate cyclase, and while progesterone significantly inhibits both F⁻ and Gpp(NH)p-stimulated activity, there is no inhibition of activity measured in the presence of manganese. These results indicate that progesterone inhibits the oocyte adenylate cyclase by directly or indirectly acting on the guanine nucleotide regulatory protein subunit of adenylate cyclase.

As isolated from the ovary, ripe Xenopus oocytes are physiologically arrested in first meiotic prophase. When incubated with progesterone, the oocytes resume meiotic cell division and progress to second meiotic metaphase, where they remain until fertilization or activation. A variety of evidence (1, for review) indicates that progesterone triggers entry into division by interacting with the cell surface rather than penetrating the cell and interacting with a cytosolic receptor, as in the classical method of steroid action. The evidence includes experiments in which steroid bound to a polymer is able to induce division while microinjection of free or polymer-bound steroid has no effect (2-4). Other evidence for the importance of a cell surface interaction comes from experiments in which continuous exposure of oocytes to high concentrations of local anesthetics or other membrane perturbants mimics the effect of progesterone (5).

Several intracellular changes following progesterone administration have been measured to elucidate the regulatory systems in the oocyte that respond to steroid/surface interaction and trigger cell division. In Xenopus, Maller et al. (6) measured a dramatic fall in intracellular cAMP levels to 40-60% of basal within several minutes after progesterone treatment, suggesting that decreased levels of cAMP may be a direct result of progesterone action. Similar results were obtained in other laboratories and with oocytes from different species (7-9). Studies by Maller and Krebs (10) utilizing microinjection into the oocyte of the regulatory and catalytic subunits of cAMP-dependent protein kinase demonstrated that this decrease in cAMP was both necessary and sufficient to trigger cell division in the oocyte.

The observed decrease in cAMP could be achieved by inhibition of adenylate cyclase, activation of phosphodiesterase, excretion, or a combination of these mechanisms. In the case of Xenopus, excretion has been eliminated as a possible mechanism by monitoring cAMP in the medium of oocytes treated with progesterone (6). In this and other laboratories (6, 9) cholera toxin, an irreversible activator of adenylate cyclase in eucaryotic cells, was shown to be a potent inhibitor of progesterone-induced cell division, with half-maximal inhibition at a toxin concentration of 8 pm (6). In addition, microinjection of the A subunit of cholera toxin was also found to inhibit subsequent progesterone-induced cell division (6). The observed decrease in cAMP combined with the inhibition of division by cholera toxin suggested that the activity of adenylate cyclase could be involved in the mechanism of progesterone action.

This possibility was strengthened when Mulner et al. (11) reported that progesterone could inhibit the in vivo conversion of microinjected [α-32P]ATP to cAMP and correlated the extent of inhibition of cAMP synthesis with the concentration of progesterone and the frequency of germinal vesicle (nuclear) breakdown. However, synthesis of cAMP in the intact oocyte could not be detected in the absence of phosphodiesterase inhibitors, and the low activity reported suggested that the injected ATP might not be equilibrated with the ATP pool used by the cyclase (1, 11). However, other studies showed that the total level of extractable cAMP in cholera toxin-treated oocytes was reduced if progesterone was present during toxin activation (9).

Another question concerns which component of the adenylate cyclase system is involved in the inhibition by progesterone.
Adenylate cyclase activity was determined by measuring the enzymatic conversion of $[\alpha-32P]ATP$ to $[\alpha-32P]AMP$. $[\alpha-32P]ATP$ was synthesized by the method of Johnson and Walseth (22). Product formation was measured by isolation of $[\gamma-32P]AMP$ using a modification of the method of Salomon et al. (23), as described by Minneman et al. (24). The reaction was carried out in a final volume of 0.2 ml containing washed membrane pellets (40-240 µg of protein) resuspended in 50 mM Tris-maleate (pH 7.5), 0.25 mM ATP, 1.5 mM Mg acetate, 0.5 mM EGTA, 5 mM cAMP, 5 mM ATP, and 10% sodium dodecyl sulfate, and the reaction tubes were heated in a boiling water bath for 10 min to solubilize membrane protein. Sample volumes were then increased to 1.0 ml with deionized water prior to chromatography over consecutive Dowex and alumina columns (23). An estimation of cyclic nucleotide recovery was routinely obtained by chromatography of a known amount of $[\gamma-32P]AMP$, and recovery averaged 60% in most experiments. All values shown have been corrected for recovery. The Student’s t test was used to evaluate the statistical significance of experimental results.

**RESULTS**

**Adenylate Cyclase Activation by Cholera Toxin**—Initial experiments were designed to validate the assay system by measuring enzyme activity as a function of time and protein. Fig. 1 shows the linearity of basal adenylate cyclase activity in washed membrane fractions as a function of assay time. Each sample contained 10 membranes (80 µg of total protein), and with the assay conditions described under “Methods,” the production of $[\gamma-32P]AMP$ was linear for at least 4 h with a correlation coefficient of 0.954 as determined by least squares analysis. From these results, a 2-h assay time was chosen for subsequent experiments. Fig. 2 shows basal adenylate cyclase activity as a function of protein content, and the results show a linear relationship between protein content and product formation through the entire range tested ($r = 0.955$). Subsequent experiments routinely measured the enzyme activity of 10 membranes per tube (50-80 µg/tube) in triplicate at 30°C during a 2-h assay.

The adenylate cyclase activity in the *Xenopus* oocyte membrane was activated by pretreatment of the intact oocytes with cholera toxin prior to membrane isolation, in both a concentration- and time-dependent fashion. Fig. 3 shows the dose response curve for in *vitro* activation of adenylate cyclase by cholera toxin. Whole cells were preincubated for 3 h in the presence of increasing concentrations of holotoxin and then washed, after which membranes were isolated and assayed for enzyme activity. Half-maximal activation was seen with 1 µM toxin, and 10 µM cholera toxin activated adenylate cyclase to

1. The abbreviations used are: G/F subunit, guanine nucleotide regulatory protein; Gpp(NH)p, guany-5'-yl imidodiphosphate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

**EXPERIMENTAL PROCEDURES**

**Materials**

Healthy *Xenopus laevis* were obtained from the South African Snake Farm, Fish Hoek, South Africa. Progesterone was purchased from Calbiochem, and cholera toxin was from Schwarz/Mann. Bovine serum albumin freed of all growth factor activity was obtained from Reheis Chemicals. Pregnant mare’s serum gonadotropin and Gpp(NH)p were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

**Methods**

**Oocyte Dissection and Pretreatment**—Sexually mature *X. laevis* females were primed by injection of 35 IU of pregnant mare’s serum gonadotropin into the dorsal lymph sac 3 days prior to decapitation. Ovarian fragments were rinsed and incubated in a medium containing 83 mM NaCl, 1 mM MgCl₂, 0.5 mM CaCl₂, and 10 mM Heps, pH 7.9. Individual unblemished (stage VI) oocytes were manually dissected from enveloping cell layers with watchmakers’ forceps after incubation of ovaries in 110 mM NaCl, 1 mM EDTA, 10 mM Heps, pH 7.9, for 30–60 min (20). After a standard 1-h re-equilibration period, intact oocytes were treated with various concentrations of cholera toxin and progesterone as specified in the figure legends. Preincubations were performed in a final volume of 0.2 ml of medium containing 1 mg/ml of insulin-free bovine serum albumin. Preincubations were carried out at room temperature with gentle shaking, and oocytes were then washed three times in medium, and membranes were isolated and assayed. When progesterone was included with cholera toxin during the preincubation period, it was also routinely included during membrane isolation and assay, since preliminary experiments indicated maximal inhibition was observed under these conditions. In some experiments, oocytes that had been manually dissected were treated with pronase (50 µg/ml) for 6 min to remove any follicle pretreated oocytes remaining after EDTA treatment.

**Membrane Isolation**—Oocyte plasma membranes were isolated by manually opening pretreated oocytes with forceps in ice-cold isolation buffer (10 mM NaCl, 10 mM Heps, pH 7.9). After soaking for 10 min, the plasma membrane/vitelline envelope complex begins to lift away from the bulk of the cellular contents and is peeled away from the yolk and cell debris with watchmaker’s forceps, transferred to and washed in fresh ice-cold isolation buffer with a Pasteur pipette, collected by centrifugation at 10,000 × g for 5 min, and assayed for adenylate cyclase activity. The manual manipulations are facilitated by the association of the vitelline envelope of the oocyte with the membrane. The average protein content, as measured by the method of Lowry et al. (21), using bovine serum albumin as a standard, was 5 µg per membrane. Routinely, adenylate cyclase activity was measured on triplicate samples of 16 membranes per tube (50 µg of protein).

**Adenylate Cyclase Assay**—Membrane-associated adenylate cyclase activity was determined by measuring the enzymatic conversion of $[\alpha-32P]ATP$ to $[\alpha-32P]AMP$. The reaction was carried out in a final volume of 0.2 ml containing washed membrane pellets (40-240 µg of protein) resuspended in 50 mM Tris-maleate (pH 7.5), 0.25 mM ATP, 1.5 mM Mg acetate, 0.5 mM EGTA, 5 mM cAMP, 0.75 mM Mg acetate, 0.1 mg/ml of creatine phosphokinase, 10 mM creatine phosphate, 0.15 mM GTP, and 20 µCi of $[\gamma-32P]ATP$. In various experiments, certain other additions were included in the assay mixture, and these are mentioned in the figure legends. Assays were initiated by addition of ATP, and the standard incubation time was 2 h at 30°C. The reaction was terminated by addition of 0.2 ml of a solution containing 50 mM Tris-Cl (pH 7.8), 5 mM cAMP, 5 mM ATP, and 10% sodium dodecyl sulfate, and the reaction tubes were heated in a boiling water bath for 10 min to solubilize membrane protein. Sample volumes were then increased to 1.0 ml with deionized water prior to chromatography over consecutive Dowex and alumina columns (23). An estimation of cyclic nucleotide recovery was routinely obtained by chromatography of a known amount of $[\gamma-32P]AMP$, and recovery averaged 60% in most experiments. All values shown have been corrected for recovery. The Student’s t test was used to evaluate the statistical significance of experimental results.
maximal levels. In many experiments of this type, basal activity ranged from 1-5 pmol/mg/h, and cholera toxin activation resulted in maximal levels of activity 2- to 10-fold above basal. However, in all cases the relative dose response relationship between cholera toxin concentration and maximal activation was similar to that in Fig. 3.

In other cell types, the characteristic time course for cholera toxin activation of adenylate cyclase is sigmoidal, and enzyme levels rise after an initial lag due to the time required for holotoxin binding and entry of the A subunit into the cell (27). The time course for oocyte adenylate cyclase activation by cholera toxin is shown in Fig. 4. The time course displays an initial lag of 0.5 h before a significant rise of adenylate cyclase activity is seen, and the maximal activity is reached after a 1.5-h incubation with 5 nM cholera toxin.

Inhibition of Adenylate Cyclase by Progesterone—Initial
Maximal effects of progesterone seen at 10–20 μM prevented about 60% of maximal toxin stimulation. Other experiments have shown that 0.001 μM progesterone has no effect on activation of adenylate cyclase by cholera toxin, and 0.01 μM progesterone causes a slight but statistically insignificant inhibition. An important question concerns whether progesterone might inhibit activation by preventing binding of toxin or interfering with entry of the A subunit into the cell. To answer this question, the A subunit of cholera toxin was microinjected into 60 oocytes which were then divided into two groups and incubated for 2 h in the presence and absence of 10 μM progesterone. The microinjection of this amount of A subunit has been previously shown to totally inhibit subsequent progesterone-induced cell division (6). As shown in Fig. 6, the activation of adenylate cyclase by A subunit was inhibited by progesterone to an even greater extent than by holotoxin. Due to different incubation times in this experiment, a subunit-activated enzyme activity was somewhat less than that seen with holotoxin, but in other experiments A subunit has activated membrane adenylate cyclase activity to levels comparable to holotoxin. Another question concerns whether the inhibition is specific for a steroid capable of inducing cell division. As shown in Table I, the effect of progesterone on cholera toxin activation of adenylate cyclase is specific and is not seen when cells are treated with 17β-estradiol, a noninducing steroid. Similarly, when intact oocytes are microinjected with [α-32P]ATP, 17β-estradiol fails to inhibit basal cAMP synthesis under conditions where progesterone-dependent inhibition can be demonstrated (11).

These results indicate that progesterone can prevent activation of adenylate cyclase by cholera toxin. To test whether progesterone was also capable of reversing cholera toxin activation of adenylate cyclase, oocytes from the same female as that used for the experiment summarized in Table I were preincubated in 5 nM cholera toxin for 2 h prior to membrane isolation and adenylate cyclase assay as described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Time of cholera toxin preincubation</th>
<th>Subsequent progesterone treatment</th>
<th>Adenylate cyclase activity pmol/mg protein/h</th>
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<tr>
<td>h</td>
<td></td>
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<tr>
<td>0</td>
<td></td>
<td>6 ± 1</td>
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<tr>
<td>0.5</td>
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<td>7 ± 1</td>
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<td>15 ± 4</td>
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Differences were significant at p < 0.005, 0.002, 0.025, 0.001. **Expressed as mean of triplicate determinations ± S.E. CT, cholera toxin. ***Significantly different from treatment activity after pretreatment with cholera toxin alone (p < 0.05).

Effect of Progesterone on Other Activators of Adenylate Cyclase—Since the effect of progesterone on cholera toxin activation suggested an interaction with the G/F regulatory protein, the interaction of progesterone with other activators of adenylate cyclase was measured. Fig. 7 compares the effects

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**Table I**

Comparison of progesterone and 17β estradiol effects on cholera toxin activation of adenylate cyclase activity

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Adenylate cyclase activity pmol/mg protein/h</th>
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<tr>
<td>None</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Cholera toxin</td>
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<tr>
<td>CT/Progesterone</td>
<td>10 ± 1</td>
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<tr>
<td>CT/17β-estradiol</td>
<td>17 ± 1</td>
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* Expressed as mean of triplicate determinations ± S.E. **CT, cholera toxin. ***Significantly different than activity measured after pretreatment with cholera toxin alone (p < 0.05).

**Table II**

Inability of progesterone to reverse cholera toxin activation of adenylate cyclase activity

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Subsequent progesterone treatment</th>
<th>Adenylate cyclase activity pmol/mg protein/h</th>
</tr>
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<tbody>
<tr>
<td>h</td>
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<tr>
<td>0</td>
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<td>6 ± 1</td>
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<td>0.5</td>
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<td>1.0</td>
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<td>16 ± 2</td>
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<td>2.0</td>
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<td>15 ± 4</td>
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</table>

Differences were significant at p < 0.005, 0.002, 0.025, 0.001. *Expressed as mean of triplicate determinations ± S.E. CT, cholera toxin. **Significantly different than activity measured after pretreatment with cholera toxin alone (p < 0.05).
of 10 μM progesterone on basal activity, cholera toxin activation, and stimulation of enzyme activity by Gpp(NH)p, fluoride (F⁻), and manganese (Mn²⁺). In this experiment a 2.5-h preincubation with progesterone significantly inhibited basal activity by 50% (p < 0.075). Cholera toxin pretreatment activated enzyme activity to a level 4.5-fold above basal, and Gpp(NH)p and F⁻, which also interact with the G/F protein (19), stimulated enzyme activity 40- and 5-fold, respectively. Progesterone significantly inhibited the stimulation of adenylate cyclase by each of these treatments. Activity measured in the presence of 10 mM Mn²⁺, a nonphysiological activator of the catalytic subunit (16), was stimulated to a level 17.5-fold above basal, but this stimulation was not significantly affected by progesterone.

**DISCUSSION**

The results presented in this paper support the belief that steroids induce meiotic cell division in amphibian oocytes by lowering the intracellular level of cAMP. Moreover, these results indicate that all or part of the mechanism by which progesterone lowers the level of cAMP involves inhibition of adenylate cyclase activity in the plasma membrane via action on the G/F subunit or the interaction of G/F with the catalytic subunit (19). However, these results not only support the earlier evidence that indicates initial steroid action in this system occurs at the level of the plasma membrane (2-5). A considerable body of evidence has accumulated in a variety of experimental systems which demonstrates that hormonal activation of adenylate cyclase involves effects on the interaction of the G/F protein with the catalytic subunit of adenylate cyclase (12). Other evidence has suggested that a number of neurotransmitters including opiates, dopamine, catecholamines (a-adrenergic), and cholinergic agents (muscarinic), as well as adenosine, angiotensin II, prostaglandin E₁ and E₂, and nicotinic acid act all or in part by inhibition of adenylate cyclase in various cell systems (28-32). This inhibition also appears to be mediated by the G/F protein. All of these inhibitory hormonal effects on adenylate cyclase share several common features, including inhibition of both basal and hormone-stimulated activity to the same extent (40-60%; Ref. 32). The evidence presented in this paper indicates that progesterone can only inhibit activation of adenylate cyclase by agents which have been shown to act on the G/F protein; thus induction of meiotic cell division in amphibian oocytes by progesterone may provide another example of a physiological regulator acting via the G/F protein to inhibit adenylate cyclase. The fact that progesterone can also significantly inhibit basal activity (Fig. 7) suggests that the G/F protein may be required for the expression of basal as well as stimulated activity. The basal activity of the oocyte adenylate cyclase is low although within the range previously reported for other nonmammalian eucaryotes. The level of basal activity suggests that the low activity seen in vivo with microinjected [α-32P]ATP is not a result of nonequilibration of injected label with the endogenous ATP pool (1, 11). It is not possible at present to determine whether the inhibition of basal adenylate cyclase by progesterone results in enough of a decrease in total intracellular cAMP to trigger division in the oocyte or whether an additional activation of phosphodiesterase is involved. On the one hand, the basal specific activity of the cyclase (0.05 pmol/oocyte/h) seems too low to account totally for the observed decrease in cAMP of about 0.4 pmol/oocyte/5 min (6) but, on the other hand, cholera toxin prods to be a potent inhibitor of cell division at a concentration of 8 M (6), conditions where basal activity is elevated only 2-fold (Fig. 4). It is possible that only a small change in cAMP levels is sufficient to trigger the oocyte to divide, as suggested by the fact that microinjected protein kinase subunits control the process at the level of 0.1 pmol/oocyte (10) and that in other cells the regulatory concentration of cAMP is close to that of the regulatory concentration of protein kinase (33). In this connection, it should be noted that much of the extractable cAMP in oocytes (1.2 pmol/oocyte, Ref. 5) is present in the yolk storage compartment and may not participate in regulation at this stage of development (1).

The conclusion in this paper that the inhibition of adenylate cyclase by progesterone involves the G/F subunit is based upon genetic and biochemical evidence accumulated in other systems, both mammalian and nonmammalian, indicating that Gpp(NH)p, F⁻, and cholera toxin all stimulate adenylate cyclase activity by interacting with the G/F protein, while Mn²⁺ affects the catalytic subunit. Progesterone could inhibit adenylate cyclase in the oocyte membrane by a number of mechanisms, including an effect on the G/F protein via binding of hormone to a specific receptor, an effect on catalytic subunit limiting the interaction of G/F protein with catalytic subunit but not the stimulation of catalytic subunit by Mn²⁺, or general membrane perturbation which might nonspecifically alter the interaction of adenylate cyclase subunits. The first of these proposed mechanisms seems most likely, since the inhibitory effect of progesterone is specific and not seen with 17β-oestradiol, a noninducing steroid which should display any nonspecific steroid membrane perturbations (Table I). Further support for the first possibility comes from preliminary photoaffinity labeling experiments that suggest the presence of a steroid receptor on the oocyte membrane. However, to distinguish these possibilities unequivocally, it will be necessary to further characterize the subunit structure of the oocyte adenylate cyclase.

A characterization of the G/F would be of more interest in relation to the results in this paper since this might contribute to a more detailed understanding of how progesterone inhibits cholera toxin action. It seems clear from the A subunit injection experiments that progesterone does not prevent cholera toxin binding or A subunit internalization. It is not clear why progesterone can totally inhibit activation by microinjected A subunit but only partially the activation by externally applied holotoxin (Fig. 5). Similar results have been obtained with pronase-treated oocytes, eliminating the possibility that the activity resistant to inhibition is due to residual follicle cells. It is intriguing to speculate that the binding of toxin to its receptor decreases the availability of the G/F protein for the inhibitory action of progesterone, and this possibility can be tested by measuring the ability of progesterone to inhibit activity by microinjected A subunit in the presence and absence of externally applied purified B subunit.

Rodbell (34) has presented a model in which the receptors and G/F regulatory proteins of adenylate cyclase exist in the basal state as an aggregate separate from the catalytic subunit. The results presented in this paper are consistent with this hypothesis. However, further work is needed in order to fully evaluate the model in relation to the oocyte system.

Acknowledgments—We thank Dr. Barry Wolfe for stimulating discussions during the course of this work.

**REFERENCES**


S. Sadler and J. Maller, manuscript in preparation.
Inhibition of Oocyte Adenylate Cyclase by Progesterone