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CALCIUM-GONADOTROPIN INTERACTION AND THEIR REGULATION OF ADENYLATE CYCLASE IN RAT LUTEAL MEMBRANES

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ABSTRACT

Adenylate cyclase in purified rat luteal plasma membrane preparations was exquisitively sensitive to calcium(Ca^{+2}) inhibition in both basal and luteinizing hormone(LH)-stimulated states. The objective of this thesis is to investigate the mechanism of Ca^{+2} inhibition of luteal adenylate cyclase.

Ca^{+2} in micromolar concentrations inhibited magnesium (Mg^{+2})-dependent luteal adenylate cyclase with a half maximal inhibition of 10-20uM. In addition, Ca^{+2} in micromolar concentrations also inhibited fluoride-, guanosine triphosphate(GTP)-, and guanyl-5'-yl-imidodiphosphate(GppNHp)-stimulated luteal adenylate cyclase activity. Inhibition of adenylate cyclase activity by preincubation with Ca^{+2} at concentrations of 2.5-100uM was reversible when 5mM EDTA was subsequently included in the assay. Inhibition by low concentrations of Ca^{+2}($\tilde{}\leq$2.5uM) was prevented with concentrations of GTP greater than 10mM or concentrations of GppNHp greater than 1uM but only in the presence of LH. Inhibition by Ca^{+2} at concentrations greater than 2.5uM was not prevented even in the presence of very

high concentrations of GTP or GppNHp. Addition of 20uM Ca^{+2} did not appear to interfere with the binding of guanine nucleotides to luteal membranes, but evidence indicated that Ca^{+2} may interfere with the dissociation of guanine nucleotides from the membranes.

All three divalent cations, Ca^{+2}, Mg^{+2}, and manganese (Mn^{+2}) were found to inhibit luteal adenylate cyclase activity, but the effective concentrations for their inhibition differed. Ca^{+2} was inhibitory at concentrations as low as luM while Mg^{+2} or Mn^{+2} was inhibitory only at concentrations greater than 5mM. Both Mg^{+2} and Mn^{+2} stimulated adenylate cyclase activity at concentrations greater than lmM, but only Mn^{+2} stimulation occurred in the absence of LH. Mg^{+2}-stimulated luteal adenylate cyclase was sensitive to Ca^{+2} inhibition in the micromolar range in the presence or absence of LH, while Mn^{+2}-stimulated enzyme activity was resistant to Ca^{+2} inhibition. Ca^{+2} appeared to interact in a competitive manner with Mg^{+2} but not with Mn^{+2} on luteal adenylate cyclase activity; increasing Mg^{+2} concentrations led to an attenuation of Ca^{+2}-inhibition of the enzyme activity.

The present result showed luteal adenylate cyclase activity was inhibited by Ca^{+2} in micromolar concentrations that would be attainable under physiological conditions. This inhibitory effect of Ca^{+2} was seen in the presence of various adenylate cyclase stimulatory agents with the exception of Mn^{+2}. Inhibition of LH-stimulated enzyme activity by concentrations of Ca^{+2} less than 2.5uM was prevented by GTP and GppNHp but inhibition by higher concentrations of Ca^{+2} was not blocked by guanine nucleotides. Ca^{+2} did not inhibit Mn^{+2}-sensitive enzyme activity at any concentration. Several modes of interaction between Ca^{+2} and luteal adenylate cyclase are possible. One site of Ca^{+2} action may be on the regulatory protein(G) because inhibition with low concentrations of Ca^{+2}($\leq$2.5uM)] was reversed by GppNHp. Another action of Ca^{+2} may be on a Mg^{+2}-sensitive site of adenylate cyclase since Mg^{+2} and Ca^{+2} showed competitive inhibition. Ca^{+2} may also act directly on the catalytic subunit site because Ca^{+2} inhibition at concentrations greater than 2.5uM was not prevented by guanine nucleotides and Ca^{+2} at all concentrations tested did not inhibited Mn^{+2}-sensitive enzyme activity. In addition, evidence for the existence of an inhibitory

regulatory subunit(G_i) in luteal adenylate cyclase was presented. Although a possible role for Ca^{+2} activation of G_i remains speculative, Ca^{+2} could conceivably regulate G_i activation resulting in inhibition of adenylate cyclase. Nevertheless, adenylate cyclase activity was shown to be exquisitively sensitive to inhibition by very low and physiologically relevant concentrations of Ca^{+2}. We, therefore, propose that Ca^{+2} is an important inhibitory cation in the functional regulation of the responses of the luteal cells to LH.

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Deep appreciation to Dr. Harold R. Behrman, my thesis advisor, for his outstanding guidance, inspiration and support. I wish to also thank members of Dr. Behrman's laboratory and Dr. Ray Aten for their assistance.

This work is dedicated to my parents,
for all their love,
motivation, and
encouragement.

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INTRODUCTION

In every estrous or menstrual cycle, highly integrated endocrine events are necessary for the development and the ultimate rupture of the ovarian follicle(s), the release of a mature oocyte, and formation of a corpus luteum from the remnants of the ruptured follicle. Differentiation and regression of the corpus luteum is characteristic of all mammalians species, and is highly endocrine-regulated and controlled. The nature of the hormonal requirements of the corpus luteum varies among species, but LH seems to be a common component of the luteotropic hormone complex in all species. In the rat, another hormone which appears to support luteal function in conjunction with LH is prolactin, whereas in the human, LH appears to be the predominant luteotropic hormone.

The endocrine maintenance of the corpus luteum during the nonpregnant as well as the pregnant cycle differs according to species and has been reviewed recently by Hammerstein et al.(1). In the nonpregnant state of the rat, there are two types of luteal phases. An "incomplete" luteal phase that is short, ranging from 1-2 days. During this "incomplete" luteal phase, the corpus luteum of the estrous cycle secretes a small amount of progesterone for

one day after ovulation, but the levels decline on the following day unless differentiated into a "complete" luteal phase either by mating or by cervical stimulation. In the rat this stimulus evokes two daily surges of prolactin secretion by the anterior pituitary(2). The prolactin surges rescue the corpus luteum and elevates progestin secretion. LH is required for progesterone secretion since administration of LH antiserum can cause luteal regression at this time(3, 4). The rat corpus luteum requires an intact pituitary to maintain steroidogenesis, for prolactin action early in the life of the rat corpus luteum is necessary for acquisition of LH receptors(4) as well as luteal estrogen receptors(5).

It is generally accepted that the steroidogenic action of luteinizing hormone(LH) in the luteal cell is mediated by an adenylate cyclase linked mechanism(6). In essence, LH binds to a membrane receptor, stimulates adenylate cyclase, and leads to cyclic AMP(cAMP) accumulation by the corpus luteum. Addition of both exogenous cAMP and phosphodiesterase inhibitors also stimulate luteal steroidogenesis(6). cAMP in the cell is thought to bind to protein kinase, and a number of intracellular events are initiated, a principal one being an increase in progesterone secretion(7). LH was shown to determine the rate and extent of progesterone production in a direct, dose-dependent

fashion in vivo(8). Rat luteal cells in culture show a time- and dose-dependent increase in their responsiveness to LH with cAMP accumulation and progesterone synthesis(9). Although cAMP mimics the steroidogenic effects of LH, the precise relationship between LH, cAMP, and luteal steroidogenesis remains unclear. Several investigators have shown that LH stimulated cAMP-dependent protein phosphorylations may lead to an increase in the availability of free cholesterol as substrate for mitochondrial pregnenolone production(10), and in the activities of the cholesterol side chain cleavage reaction, the rate limiting reaction in steroidogenesis(10, 11).

Corpus luteum function is transient and is dependent on gonadotropin support for continued function. Regression of the corpus luteum, or luteolysis is marked by a sharp decrease in serum progesterone and increased secretion of its metabolite 20a-hydroxyprogesterone. Biochemical signs of luteolysis are followed by histological changes indicative of organ involution(9). Although the physiological cause(s) of luteolysis are unclear, gonadotropin support is apparently crucial for the function of the corpus luteum(12) and an interruption of gonadotropin support may induce luteolysis. A decrease in the availability of gonadotropin is not involved in the initiation of luteolysis since direct measurements of

circulating levels of gonadotropin show that no decrease precedes luteal regression(9). Since the serum levels of LH and prolactin are not decreased at the time of physiological luteolysis, local factors must be responsible for the early stages of luteolysis. The LH receptor content probably ultimately determines the ability of the corpus luteum to survive and down regulation of LH receptors is associated with luteolysis(13). A decrease in LH receptors or down regulation could be one mechanism for rendering the cells insensitive to gonadotropins, but this event is preceded many hours by biochemical changes that mark the beginning of physiological luteolysis(14). If prostaglandin(PG) was injected into a pseudopregnant rat, the inhibitory effect on luteal progesterone secretion was more rapid than a later decline in LH receptors, suggesting that the luteolytic action of PG was not mediated directly by a decrease in LH receptors(14).

Although the physiological mechanism of luteal regression is unclear, luteolysis in many species can be induced by administration of prostaglandin F_{2a}(PGF_{2a})(15, 16). There is now abundant evidence that implicate PGF_{2a} as the natural luteolysin in many species, including pigs, cows, guinea pigs, sheeps, rats, monkey(1, 16-19)--with possibly one exception, the human(20). Evidence for a physiological role of PGF_{2a} in corpus luteum regression is

based upon several observations. First, injection of lipid-soluble extracts of uterine endometrium, which were later identified to contain prostaglandin F_{2a}, was found to cause luteolysis(19). Second, direct administration of PGF_{2a} cause luteal regression in a wide variety of species(12, 16). Third, active immunization of the animal against PGF_{2a} or removal of the uterus in the animal prolongs the life span of the corpus luteum(21). Fourth, inhibition of PG synthesis with indomethacin prolongs the life span of the corpus luteum(21). Fifth, an increase in the blood level of PGF_{2a} occurs at or near the time of luteal regression(16). Evidence in support of a physiological role of PGF_{2a} in corpus luteum regression has been reviewed(15, 16, 22). PGF_{2a} is thought to be synthesized in the uterus and transferred to the ovary via counter-current exchange between the uterine vein and ovarian artery(16). There is recent evidence to suggest that the rat corpus luteum can synthesize prostaglandins which may be involved in the auto-regulation of its own function(23,24).

Initially, several mechanisms were postulated for the luteolytic action of PGF_{2a}. These include restriction of blood supply to the ovary(25) or corpus luteum(26), stimulation of a lytic level of LH secretion from the

pituitary(27), or antagonism of gonadotropin support of the corpus luteum(28). Currently, it is thought that the site of action of PGF_{2a} in luteolysis is directly on the corpus luteum(28, 29), as an antagonist of gonadotropin support. This conclusion is based upon observations which showed that PGF_{2a} is also luteolytic in hypophysectomized rats in which the corpora lutea were maintained with exogenous gonadotropin(15, 16). Thus stimulation or inhibition of pituitary hormone secretion appears not to play a major role in PGF_{2a}-induced luteolysis. PGF_{2a} does not appear to cause luteal regression by reducing blood flow to the corpus luteum since any significant changes in luteal blood flow to the corpus luteum is preceded by a decrease in progesterone secretion. This was shown in the rat(30), rabbit(31), ewe(32); intra-arterial administration of PGF_{2a} did not change blood flow to the corpus luteum for many hours after plasma progesterone was significantly reduced. Thus, PGF_{2a} appears to act directly on the corpus luteum and rapidly antagonizes LH-stimulated cAMP accumulation and progesterone synthesis. Grinwich *et al.* initially demonstrated that incubating rat luteal slices with PGF_{2a} and LH diminished the rise in cAMP seen with LH alone(33). Lahav *et al.* later confirmed these results using intact corpora lutea in culture(34). In isolated luteal cells, PGF_{2a} stimulates

basal progesterone accumulation but antagonizes the ability of LH to increase cAMP and progesterone accumulation (7)without affecting the binding of ^{125}I-HCG to luteal cells(35). PGF_{2a} also diminishes progesterone accumulation in response to dibutyl cAMP ın luteal cells, indicating that PGF_{2a} inhibits steroidogenesis at two loci: formation of cAMP and step(s) distal to cAMP accumulation(21, 36).

Another factor shown to modulate the response of the corpus luteum to LH and thereby to control the functional state of this gland is gonadotropin releasing hormone(LHRH)(37). There is substantial evidence to support the conclusion that LHRH, like PGF_{2a}, is a luteolytic agent which suppresses corpus luteum function(37). LHRH acutely antagonizes LH-dependent cAMP accumulation in a manner similar to, but independent of, PGF_{2a}; unlike PGF_{2a}, dibutyryl cAMP reverses the inhibition in progesterone synthesis by LHRH(38). In the rat, both luteolytic agents have a direct effect on the luteal cell in vitro(39), specific receptors are present in the cells for PGF_2 (40) and LHRH(38), and their acute affect is to block the stimulatory response to LH. The very early action of both PGF and LHRH(38) in the luteal cells is inhibition of LH-sensitive cAMP accumulation by a mechanism that is

independent of an effect on LH receptor-binding activity, cAMP degradation, or a direct action on adenylate cyclase. Consequently, it appears that PGF_{2a} and LHRH interfere with activiation of adenylate cyclase by the occupied receptor. Since the inhibitory effects of both prostaglandins F_{2a} and LHRH are identical, they occur only in the intact cell, and they do not directly inhibit adenylate cyclase activity in luteal membranes, it is likely that their effects are receptor-mediated and that a common intracellular second messenger may mediate the actions of both agents. Behrman et al.(18) proposed that Ca^{+2} is the possible mediator of PGF_{2a} or LHRH in the luteal cell. When dispersed luteal cells were incubated in media depleted of Ca^{+2}, an increase in LH-stimulated cAMP accumulation of about two fold was seen compared to the same response in medium that contained 1.8 mM Ca^{+2}(41). Addition of calcium ionophore(A23187) to dispersed luteal cells inhibited LH stimulation of adenylate cyclase in a dose-dependent manner comparable to PGF_{2a} and LHRH(41), and inhibition was dependent on the presence of extracellular Ca^{+2}. Also, A23187 significantly inhibited LH-stimulated progesterone secretion in response to cholera toxin and inhibited cholera toxin-stimulated cAMP accumulation(41). Ca^{+2} and/or A23187 did not affect LH receptor-binding activity or cAMP degradation(41). The

inhibition of LH-sensitive adenylate cyclase by Ca^{+2} was seen both in intact luteal cells and in luteal membrane preparation. These studies suggested that increased intracellular Ca^{+2} mimics the inhibition of LH stimulated cAMP accumulation by PGF_{2a} and LHRH.

Additional support for the role of Ca^{+2} in regulating luteal adenylate cyclase has come from studies with ouabain and monensin(42), these drugs increase intracellular levels of Na^{+1} by inhibition of Na^{+1} extrusion and by a direct ionophore effect, respectively. In the presence of extracellular Na^{+1} and Ca^{+2}, both drugs produce a marked and dose-related inhibition of LH-stimulated cAMP accumulation and progesterone secretion identical to that seen with PGF_{2a} and LHRH in intact luteal cells without affecting LH receptor binding activity or cAMP degradation(42). Removal of Na^{+1} and/or Ca^{+2} from the extracellular medium abolishes drug-dependent inhibition on luteal cAMP accumulation(42); reducing extracellular Na^{+1} or using tetrodotoxin, a Na^{+1} channel blocker, also eliminates this effect(42). Ouabain and monensin probably produce a Na^{+1}-dependent influx of Ca^{+2} into the luteal cell which prevents activation of adenylate cyclase by LH(42). LHRH and PGF_{2a} do not require the presence of extracellular Ca^{+2} for their

antigonadotropic action; removing extracellular Ca^{+2} or using a Ca^{+2} channel blocker, Verapamil, has no effect on the action of these luteolytic substances(42). This implies that the cell may mobilize intracellular Ca^{+2} in response to luteolytic hormones. Based on studies in which the concentration of free Ca^{+2} required to elicit LH-dependent cAMP inhibition was determined(42), release of intracellular Ca^{+2} from sequestered sites would suffice to illicit such inhibition. Although the antigonadotropin effects of the divalent- and mono-valent ionophores were dependent on the presence of extracellular Ca^{+2}(41, 42). Ouabain, monesin, and A23127 have also been reported to induce Ca^{+2} release from intracellular stores under physiological condition(43-45).

Treatment of animals or cells with PGF_{2a} and LHRH inhibits high affinity Ca^{+2}-ATPase activity in microsomes but not in plasma membranes(46). This could lead to a decrease in sequestration of Ca^{+2} into microsomes and consequently produce a rise in intracellular Ca^{+2}. Both PGF_{2a} and LHRH causes a rapid and marked increase in ^{32}P-P_i incorporation into phosphatidylinositol(PI) and phosphatidic acid(PA) in rat luteal cells in cultures(47). Since

phosphatidic acid has been suggested as a Ca^{+2} ionophore in biologic membrane(48) and lysosomes(49), changes in PI-PA metabolism may be actively involved in intracellular Ca^{+2} interaction in the adenylate cyclase system. Overall, the manner in which these luteolytic substances may release sequestered intracellular Ca^{+2} is unclear.

The rat luteal adenylate cyclase system appears to be quite similar to other mammalian cyclase systems. Birnbaumer et al. reported that corpora lutea of rabbits, rats and other species(50-52) respond to LH, to prostaglandins(PGs), and to catecholamines through stimulation of adenylate cyclase activity. The luteal adenylate cyclase system activated by these hormones appears to be the same(53) since their action on adenylate cyclase is not additive. He also showed that guanine nucleotide(54), and Mg^{+2}(53) requirements for stimulation of adenylate cyclase by these hormones do not differ substantially from those seen in other non-ovarian adenylate cyclase systems. Current adenylate cyclase models center on a three component system: separate receptors(R) for each hormone, hormone-occupation of the receptors governs the effects of guanine nucleotides acting at nucleotide regulatory proteins(G), the latter govern either stimulation(s) or inhibition(i) through distinct processes or components that affect a common adenylate cyclase unit,

the catalytic(C) unit(55). R has been shown to be a separate component by biochemical and genetic and developmental studies(56). The G unit is an intrinsic membrane protein located on the cytoplasmic side of the plasma membrane that has been purified(56). C is an intrinsic membrane protein located on the inner surface of the plasma membrane and has not yet been well characterized(56). In addition to separate R, N, and C units, there may be separate components(M) responsible for regulation by divalent metal ions (Mg^{+2}, Mn^{+2} and Ca^{+2})(57, 58).

Cassel and Selinger(59) Proposed that the regulation of adenylate cyclase by guanine nucleotides involves a cycle in which the enzyme is activate by GTP, GTP is then hydrolyzed to GDP at the regulatory site and the dissociation of the formed GDP from the regulatory site is the rate limiting step in the subsequent action of GTP. This view is based on experiments carried out with turkey erythrocyte membranes which showed the existence of GTPase activity(60) and that hormonal stimulation is associated with an increased rate of guanine nucleotide exchange at a site thought to be the adenylyl cyclase regulatory site(59). Studies on the general requirement of guanine nucleotides in hormonal stimulation of adenylate cyclase has led to the finding that various stable analogs of GTP, such as Gpp(NH)p , $Gpp(CH_2)p$,

and GTP-ψ-S, are potent activators of all hormone-sensitive adenylate cyclases; stimulatory hormones accelerate and potentiate the activation by these nucleotides(42). Their stimulatory effect is antagonized by GDP(61) or its stable analog, GDPβS(61). Fluoride is another ubiquitously stimulatory ligand of eukaryotic adenylate cyclase(62). Activation usually requires greater than millimolar concentrations of fluoride and is irreversible or only slowly reversible(62).

Recently, studies using toxins from Vibrio Cholera and Bordetella Pertussis resulted in identification and purification of the guanine nucleotide regulatory systems into two separate components, one responsible for stimulating(G_s) and the other inhibiting(G_i) adenylate cyclase(63). Hormone receptors, previously classified mostly on the basis of the actions of selective agonists and antagonists, can now also be classified by whether they operate by regulating $G_s$or G_i(64). There is evidence for the existence of both a G_s and a G_i components in the rabbit luteal adenylate cyclase(65). Both G_s and G_i activation are modulated by guanine nucleotides and both apparently have a requirement for Mg^{+2} for activation(66-68). Also, both G_s and G_i apparently possess a GTP-hydrolyzing systems(69). Currently G_s has been purified only from rabbit liver(70,

71), turkey erythrocytes(72), and human erythrocytes(73, 74), and has been found to contain an α, β(70-74) and possibly a ψ (75) subunits. The α subunits of both G_s and G_i appear to be dissimilar but both bind GTP and its analogs(76) and are substrates for ADP-ribosylation by Cholera toxins and Pertusis toxins respectively(70, 74, 75). The β subunits of the two proteins are indistinguishable by two-dimensional peptide map analysis(74) and by amino acid composition(76). The ψ subunits co-migrate on SDS-polyacrylamide and urea gradient gel electrophoresis but it is unclear whether they are the same for G_s and G_i. Gilman and collaborators(63, 71, 77-80) have suggested in the rat liver system that hormone activation of adenylate cyclase may coincide with the dissociation of α and β subunits into isolated components, with concomitant formation of high affinity complexes between the α subunits and guanine nucleotides(77). Currently, the mechanism for regulation of the equilibria between the subunits of G_s and G_i by hormone receptors or the mechanism of their interaction with the catalytic subunit in the bilayer are still speculative.

The role of the divalent ions in the adenylate cyclases stimulation has been controversial. Mg^{+2}, in addition to combining with ATP to act as substrate for the catalytic site, was also thought to cause activation of the enzyme

system through an allosteric site(81). Subsequently deHaen(82) and others(83, 84) suggested that the role of Mg^{+2} in activation of cyclase was to reduce the free protonated species of ATP in the medium which was thought to be a potent, competitive inhibitor of adenylate cyclase at the catalytic site. At a later time, Johnson et al.(85) and Londos and Preston(57) showed by kinetic analysis of a number of adenylate cyclase systems that Mg^{+2} stimulates the cyclase system allosterically but they did not identify the putative allosteric site. Studies from Birnbaumer et al.(67) also showed Mg^{+2} accelerated the responsiveness of adenylate cyclase and suggested that this allosteric site was likely to be on the regulatory subunit. Their group further suggested the mechanism of interaction between Mg^{+2} and the regulatory subunit was other than facilitating the dissociation of inhibitory GDP or stimulating the association rate of nucleotide to the system.

Variable results have been obtained using Mn^{+2} to stimulate adenylate cyclase activity. Mn^{+2} can substitute for Mg^{+2} with variable degree of efficacy in some adenylate cyclase systems. Some investigators have found Mn^{+2} to be more potent than Mg^{+2}(86, 87), others have found it to be less active than Mg^{+2}(87, 88). Although Mn^{+2} appears to be

physiologically relevant in only a few adenylate cyclase systems(87), data from cells lacking a functioning G_s showed that while Mg-ATP was not a substrate for adenylate cyclase in the presence or absence of guanine nucleotide, fluoride or hormone(87), Mn-ATP could be used as a substrate independent of guanine nucleotide, fluoride or hormone modulation. Studies based on soluble cyclase suggested that the bare catalytic unit, when alone, can only utilize Mn-ATP as substrate(87). The physiological role of Mn^{+2} as a possible regulatory ligand of receptor-cyclase function remains to be investigated.

Ca^{+2} has been shown to inhibit adenylate cyclase activity in a variety of different cells(89-93) and appears to be involved in the response to PGs(47) and LHRH(94) in other tissues. In the pituitary, several investigators have reported that Ca^{+2} mediates the action of LHRH, leading to a release of LH(94). Berridge et al.(95) suggested that most cellular processes are activated through Ca^{+2}, whereas cAMP functions indirectly to modulate these Ca^{+2}- dependent events. Free cytosolic [Ca^{+2}] in cells at rest is usually between 0.05uM to 0.5uM(44). When cells are stimulated, a transient rise in free cytosolic [Ca^{+2}] between 1 to 2uM(44) or as high as 10 to 50uM(95) have been reported.

In the studies from our laboratory by Behrman et al.,

Ca^{+2} was shown to directly inhibit activation of adenylate cyclase by LH in luteal membranes without affecting LH receptor binding activity or cyclic AMP degradation(41, 96). This implies that Ca^{+2} blocks interaction of the occupied receptor with adenylate cyclase and thereby prevents enzyme activation in a manner identical to that produced by PGF_{2a} and LHRH. Ca^{+2} produces this effect directly on luteal membranes whereas an intact cell is necessary to elicit this effect with PGF_{2a}, LHRH or with other agents that directly increase intracellular Ca^{+2} concentration. The possible site(s) or mechanism of Ca^{+2} inhibition of adenylate cyclase is unclear. Studies from Behrman et al.(96) showed that adenylate cyclase was inhibited by low concentration of Ca^{+2}(luM) which could be completely reversed by GTP in the presence of LH. This suggested that Ca^{+2} may affect GTP binding proteins that are necessary for activation of adenylate cyclase by hormone. This does not preclude other modes of interaction that an elevated intracellular Ca^{+2} may inhibit LH-stimulated cAMP increase or mediate antigonadotropic effects. Also whether the effect of Ca^{+2} on luteal adenylate cyclase is mediated through a cadmodulin regulating system is currently unclear.

The experiments in this thesis were designed to further

elucidate the nature of Ca^{+2}-inhibition of luteal adenylate cyclase. Based on the model of Selinger and Cassel(59), it is possible that Ca^{+2} may interfere with the interaction of GTP with the guanine regulatory proteins in the adenylate cyclase complex; conceivably, Ca^{+2} could interfere with either the affinity of GTP binding to the regulatory component(G), the hydrolysis of GTP by GTPase on G, or the dissociation of GDP, an antagonist for the activation of the regulatory component, from G. Attempts were made to examine the effect of Ca^{+2} on the interaction of GTP and G protein in luteal membrane preparations. The reversibility of Ca^{+2}-inhibition of luteal adenylate cyclase in isolated membrane fractions by GTP, or stable GTP analogs, or fluoride were examined. Metal ions play a critical role in determining the relative stimulation that can be elicited in the luteal adenylate cyclase system(97), one objective was also to examine the effect of Mg^{+2} or Mn^{+2} on inhibition of luteal adenylate cyclase by Ca^{+2}; which may indicate other modes of Ca^{+2} interaction in the luteal adenylate cyclase system.

MATERIALS and METHODS

ANIMALS

Immature (26-day-old) female rats (Crl:CD(SD)BR strain of Sprague-Dawley, Charles River Laboratories, Wilmington, MA) were given a single subcutaneous injection of 50 IU Pregnant Mare Serum Gonadotropin (Gestyl, Organon Pharmaceuticals, West Orange, NJ), Followed 60h later by a second injection of 25 IU human Chorionic Gonadotropin(hCG) (A.P.L., Ayerst Laboratories, Rouses Point, N.Y.). The rats were sacrificed by cervical dislocation 5-6 days after hCG injection. Their ovaries were quickly removed, used immediately or frozen rapidly with dry ice and stored at -80C. Injection of hCG, after priming with PMSG, results in extensive luteinization of ovaries(98-100).

PLASMA MEMBRANE PREPARATION

The procedure for isolation of enriched heavy plasma membranes and light plasma membranes from rat luteal tissue was based on that of Bramley and Ryan(99-101). Luteinized ovaries, freed of fat and connective tissues, were blotted dry, weighed, minced, and homogenized (10 volume/g) in 250mM

sucrose, 1 mM EDTA, 10mM Tris-HCl buffer, pH 7.4, using 10 complete strokes of a loose Dounce homogenizer. After filtering through several layers of wet cheesecloth, the homogenate was subjected to differential centrifugation. A 1,000 x g pellet and a 20,000 x g pellet (from the 1,000 x g supernatant fraction) were prepared and used for isolation of heavy and light plasma membrane fractions, respectively. All sucrose solutions used in this procedure were prepared in 10 mM Tris-HCl buffer, pH 7.4, containing 1mM EDTA and their concentration adjusted exactly prior to use with an Abbe refractometer. The 1,000 x g pellet was resuspended in buffer used for initial homogenization and layered on the top of a discontinuous sucrose gradient containing 5ml of 30%, 8ml of 36%, 8ml of 40%, and 5ml of 50% sucrose solution. The 20,000 x g pellet resuspended in buffer used for initial homogenization was layered on top of a continuous sucrose gradient, prepared using 15 ml of 20 % and 15 ml of 55% sucrose solutions. Both gradients were centrifuged at 63,000 x g for 4 h in a Beckman SW28 rotor. A heavy membrane fraction was obtained from the materials accumulating at the interface between 30% and 36% sucrose of the discontinuous sucrose gradient. A light membrane fraction was obtained from membranes in the continuous sucrose gradient which accumulated between 27% to 33% sucrose concentration inclusively. All fractions were collected with a meniscus-sensitive probe (Buchler Auto

Densi-Flow II2). The heavy and light membrane fractions, each diluted four times with buffer containing 10 mM Tris-HCl, pH 7.4 and 1mM EDTA, were pelleted at 63,000 x g overnight, resuspended in small aliquots of homogenizing buffer and stored at -80C. The heavy membrane fraction is referred to as basolateral membranes, and the light membrane fraction is referred to as microvilli membranes.

Based on studies by Bramley and Ryan(98,100), using the continuous sucrose gradient, the region between 27-35% sucrose will yield 55-70% of the total hCG-binding, but only 25-35% of the adenylate cyclase with most adenylate cyclase activity to be found between 30-40%. Therefore, collecting fractions between 27-34% will enable maximal yield of LH receptors with partial yield of adenylate cyclase activity but would ensure minimal contamination from microsomal and mitochondrial materials from the original 20,000 x g fraction.

PREPARATION OF CALCIUM SOLUTIONS AND ASSAY OF FREE CALCIUM

Analysis of the effect of Ca^{+2} on enzyme activity was determined by preparation of standard solutions of Ca^{+2} (Orion standard) which when added to the reaction mixture resulted in known concentrations of free Ca^{+2}. Free Ca^{+2} was

determined directly with an Orion Ca^{+2}-sensitive electrode(41). Calibration of the electrode was carried out by preparation of standard solution composed of variable levels of known Ca^{+2}, 25 mM Tris, and 1 mM EGTA (pH 7.5). The free Ca^{+2} level in the standard solution was calculated based on a stability constant ($K_{pH}^{Ca-EGTA}$) of 4.4×10^{6} M^{-1} in 25 mM Tris pH7.5 which was determined in this buffer(102). Results from the standard solution was used to construct a standard nomogram. The level of free Ca^{+2} in each reaction mixture was directly measured with the calibrated, calcium-sensitive electrode based on the voltage recorded which corresponded to the free calcium concentration from the standard nomogram. A nomogram of total and free calcium was established for each enzyme or receptor assay. Standard solutions of Ca^{+2} for each receptor or enzyme assay were made and stored at 4C.

PROTEIN ASSAY

Protein was assayed by the procedure of Bradford (103).

ADENYLATE CYCLASE ASSAY

Adenylate cyclase activity in membranes from luteinized

ovaries was assayed by a modification of the method of Birnbaumer et al.(51) described previously(38). Briefly, the reaction mixture (pH 7.5) contained $MgCl_2$ (5mM), EGTA (1mM), Isobutylmethyl Xanthine (0.5 mM; IBMX), Bovine Serum Albumin (1 mg/ml; BSA), Creatine Phosphate (5 mM), Creatine Phosphokinase (500 units/ml), Tris-HCL (25mM), ATP (3 mM), plasma membranes (~10ug protein), and LH (~1 ug) in a final volume of 0.1 ml was prepared at 4C. A stock solution of $MgCl_2$, EGTA, IBMX, and Tris was prepared and stored at 4C. Solutions of Creatine Phosphate, creatine phosphokinase, ATP (pH adjusted to 7.5 with KOH), and of other reagents and nucleotides were prepared immediately prior to assay. Reagents were added to tubes in an ice bath and adenylate cyclase activity was determined following incubation for 10 minutes at 37C unless otherwise specified. The reaction was stopped by incubation for 10 minute at 80C and cAMP was determined by radioimmunoassay as described(42). The standard curve of cAMP contained an equivalent volume of the reaction mixture to reduce non-specific cross reaction of the reaction mixture or reagents used.

BINDING ASSAYS

Attempts were made to determine whether Ca^{+2} affects adenylate cyclase activity by interfering with the binding

of guanyl nucleotides to adenylate cyclase subunits. Many studies have established a regulatory role for guanyl nucleotides in hormone-stimulated adenylate cyclase systems of various eukaryotic cells(61, 104-108). Since purified guanine dependent regulatory subunits were not available presently, we attempted to correlate the effect of Ca^{+2} on binding of radioactive guanine nucleotides or the nonhydrolyzable analog, Gpp(NH)p, to the light membrane fraction. The inhibitory effect of Ca^{+2} on adenylate cyclase activity when stimulated with guanine nucleotides or Gpp(NH)p was compared to binding of guanine nucleotides. The reaction mixture at pH 7.5 contained $MgCl_2$ (5mM), EGTA (1mM), IBMX (0.5mM), BSA (1mg/ml), Creatine Phosphate (5mM), Creatine Phosphokinase (500 units/ml), Tris-HCL (25mM), ATP (3mM), AppNHp (0.5mM), light membrane preparation (0.5-1mg/ml final), and various concentration of free Ca^{+2} and radioactive labelled guanine nucleotides or Gpp(NH)p. Activiation of adenylate cyclase was initiated with the addition of LH(10 ug/ml final); the reaction mixture was incubated at 30C for varying amount of time. After incubation, 0.9 ml of stopping buffer (pH 7.5 include $MgCl_2$ (5mM), EGTA (1mM), IBMX(0.5mM), Tris-HCL(25mM), GTP(1mM or 0.1mM)) $\pm Ca^{+2}$ was added to 0.1 ml of the reaction mixture and kept at 4C. Subsequently samples were then passed

through filters (HAWP 025 Millipore, 0.45 uM) under constant vacuum pressure, the filters were washed with 8 volumes of cold stopping buffer, dried and the radioactivity determined. Some of the samples prepared as above were also used to examine the amount of nucleotides bound by methods modified from that of Cassel and Selinger(59, 109). The samples were centrifuged at 12,000g x5 minutes, then washed 5 times with 1 ml of cold stopping buffer and the remaining pellet was solublized in 1% SDS. An aliquot of the sample was then counted.

MEASUREMENT OF LH-INDUCED RELEASE OF MEMBRANE BOUND ^{3}H-Gpp(NH)p

The protocol used was described by Cassel and Selinger(109). Attempts were made to examine the effect of Ca^{+2} on release of bound Gpp(NH)p in the presence of LH. Membranes were activated by ^{3}H-Gpp(NH)p with LH as described above; after incubation for 30 minutes at 30C, the samples were centrifuged at 12,000g x 5 minutes and then washed 5 times with 1 ml of cold stopping buffer until radioactivity in the supernatant fraction was at background level. The remaining pellet was resuspended in 1 ml of stopping buffer and incubated at 30C for 1 minute and then centrifuged at 12,000g x 5 minutes to remove loosely bound radioactive

material. The pellet was resuspended in a cold releasing medium containing 300ul of the original reaction mixture without radioactive substrate or additional membranes, but with 0.2 mM GTP, and LH(20ug/ml final concentration). The reaction was initiated by the addition of LH at 4C followed by incubation at 30C. Samples of equal volume were withdrawn at specific intervals and placed in 0.5 ml of ice cold stopping buffer. Subsequently the samples were centrifuged at 12,000g x 5 minutes and 200 ul of the supernatant was removed to measure ^{3}H-Gpp(NH)p released. Twenty ul of the releasing mixture was removed and assayed for cAMP content as described above. The final protein concentrations was not determined.

HORMONES, DRUGS and REAGENTS

Ovine LH (NIADDK oLH 23}, a gift from the NIH (Bethesda, MD), was dissolved in 1% BSA (1 mg/ml) and stored at -80C. GTP (Tris salt), ATP (Tris salt), and 5'-guanylylimido-diphosphate (Gpp(NH)p) were purchased from Sigma Chemical Ca. (St. Louis, MO). NaF was purchased from Fischer Chemical Co. (Pittsburgh, PA). All dry reagents were stored over dessicant at -20C unless otherwise indicated. All other reagents were purchased from Sigma chemical Co. unless otherwise indicated.

STATISTICAL ANALYSIS

Each experiment measuring adenylate cyclase activity contained three replicates for each treatment group and many of the experiment were repeated at least twice; each point represented the mean ± standard error of the mean.

IV. RESULTS

IV.1 PREPARATION OF LUTEAL MEMBRANE FRACTIONATION.

The distribution of adenylate cyclase activities associated with each fractionation is shown in Table 1. The heavy membrane fraction showed considerably less adenylate cyclase activity than the light membrane fraction. The heavy membrane pellet was further purified by ultracentrifugation on a discontinuous sucrose gradient and materials collected from the interface of the 30% and 36% sucrose gradient where adenylate cyclase activities, if present, would be enriched.

A continuous sucrose gradient enables greater resolution of distinct fractions and was used to isolated the light membrane and the microsome-enriched fractions. Both fractions showed significant basal adenylate cyclase activity and both showed an approximate four fold increase in activity following the addition of LH. The microsomal fraction contains a higher specific activity than that of the light membrane fraction.

IV.2 FREE CALCIUM DETERMINATION.

A free Ca^{+2} nomogram was developed from calibration of

an Orion Ca^{+2} sensitive electrode in a Ca^{+2}-EGTA buffer system (Figure 1). Results of the initial calibration is shown in Table 2. The electrode was calibrated using variable levels of known Ca^{+2} concentration in Tris-EGTA buffer. From the concentrations of total Ca^{+2} and EGTA in each solution, and with the $K_{pH}^{Ca\text{-}EGTA}$ developed by Bartfai et al.(102) for appropriate ionic strength and pH, the free Ca^{+2} concentrations were calculated (Table 2). This table was used as a standard for free Ca^{+2} determination in other enzyme assays. A nomogram of total and free Ca^{+2} was determined for the adenylate cyclase assay (Figure 1). Bovine serum albumin(BSA) of 1% was included in all adenylate cyclase assays, but when not included, the ratio of free Ca^{+2} to total Ca^{+2} was changed significantly (Figure2); BSA increased the concentration of free Ca^{+2}.

The presence of other metals such as Mg^{+2} and Mn^{+2} also increased free Ca^{+2} in a Ca^{+2}-EGTA buffer system by metal-EGTA chelation as described by Bartfai(102). With a concentration of Ca^{+2} at 2.6uM, when Mg^{+2} concentration was increased from 5mM to 50mM, the increase in free Ca^{+2} concentration was only 1.1uM (Table 3). With a higher Ca^{+2} concentration at 4.7uM, the total change of free Ca^{+2} was

only 1.4uM with varying the Mg^{+2} concentration from 5mM to 50mM (Table 3). The largest change in free Ca^{+2} concentrations occurred when the Mg^{+2} concentration was increased from 0 to 3mM. The changes in free Ca^{+2} concentration when Mn^{+2} concentrations were varied was of similar magnitude (results not shown). If experiments with Ca^{+2} inhibition of adenylate cyclase was performed with 0 to 3mM Mg^{+2} or 0 to 3mM Mn^{+2}, the free Ca^{+2} concentration needs to be readjusted for each Mg^{+2} or Mn^{+2} concentration. Since a constant excess of total Mg^{+2} or Mn^{+2} was used over the total amount of nucleoside-triphosphates(XTP) used in the enzyme assays, and that the $K^{Ca+2-XTP}$ was lower than that of Mg^{+2}- and Mn^{+2}-XTP, the free Ca^{+2} concentrations were relatively unchanged with XTP added to enzyme assays (Behrman et al. unpublished result).

IV.3 CA^{+2} INHIBITION OF LUTEAL ADENYLATE CYCLASE ACTIVITY.

Ca^{+2} showed a highly significant and dose-related inhibition of LH-stimulated adenylate cyclase activity (Figure 3). The addition of 0.75mM and 1.2mM in the assay mixture produced about 1 and 100uM free Ca^{+2} respectively and caused 24% and 71% inhibition of LH-stimulated cAMP accumulation.

Half-maximal inhibitory concentration (IC_{50}) of free Ca^{+2} was between 10uM and 20uM which was in concordance with earlier published work by Behrman et al.(41). In addition, the basal luteal cyclase activity in the absence of LH stimulation was inhibited by almost 40% with 100uM of free Ca^{+2} (Figure 4). Inhibition of LH-stimulated enzyme activity by Ca^{+2} was more pronounced than that of basal enzyme activity.

Addition of 5 mM EGTA prevented the inhibition by Ca^{+2} of adenylate cyclase activity (Table 4). Both the basal and LH-stimulated enzyme activity in the absence of additional Ca^{+2} was increased by the addition of 5mM EGTA. EGTA is a highly specific Ca^{+2} ion chelator, the increase in cyclase activity with the addition of EGTA probably reflected the presence of a small amount of free Ca^{+2} in the luteal membranes that prevented maximal LH stimulation. Reversal of Ca^{+2} inhibition was much more evident in the presence of LH than in its absence.

IV.4 REVERSAL Of CA^{+2} INHIBITION OF LUTEAL ADENYLATE CYCLASE BY GTP AND GPP(NH)P.

The effect of GTP and an analog of GTP, GppNHP on adenylate cyclase activity in the presence of LH and Ca^{+2}

was examined (Figure 5 - 7). In the presence of LH, a significant increase in cyclase activity was seen above 1uM GTP or 0.1uM GppNHp. GppNHp, a nonhydrolyzable analog of GTP, was a more potent activator of luteal adenylate cyclase (Figure 5). In the absence of Ca^{+2}, increasing GTP concentrations from 0 to 1,000uM resulted in a 50% increase in LH-stimulated cyclase activity (Figure 6) while raising GppNHp concentrations from 0 to 100uM resulted in a 750% increase. The maximum response to GTP or GppNHp stimulation was not tested. In the absence of hormones, GTP did not activate adenylate cyclase to the extent that was seen with GppNHp.

Inhibition of luteal adenylate cyclase by 1uM and 20uM of Ca^{+2} is shown in Figure 6 and 7. Inhibition of luteal adenylate cyclase in the presence of LH by 1uM free Ca^{+2} was reversed completely by GTP or GppNHp at concentrations of 1 to 10uM or 0.1uM respectively. Complete reversal of adenylate cyclase inhibition with 2.5uM free Ca^{+2} was also observed using 100uM GppNHp or greater but reversal was not seen with GTP at concentration as high as 1mM (data not shown). GTP or GppNHp concentrations as high as 1mM or 0.1mM respectively were unable to prevent the inhibition of adenylate cyclase by 20uM Ca^{+2}. Higher concentrations of Ca^{+2} attenuated and prevented both GTP- or GppNHp-reversal

of LH-stimulated adenylate cyclase activity.

The effect of GTP and GppNHp on adenylate cyclase activity in the absence of LH is shown in Figure 8. Increasing GTP concentrations from luM to 1,000uM resulted in an approximately 2-3 fold increase in basal adenylate cyclase activity. GppNHp at concentrations greater than 0.luM also significantly increased adenylate cyclase activity and the magnitude of rise in enzyme activity observed by GppNHp from 0 to 100uM was similar to that seen in the presence of LH. One uM of free Ca^{+2} significantly inhibited basal adenylate cyclase activity about 50% and increasing levels of either GTP or GppNHp did not reverse the inhibition of enzyme activity by luM Ca^{+2}. It appears that the presence of LH enhanced the reversal of adenylate cyclase inhibition at low concentration of free Ca^{+2}.

IV.5 INHIBITION OF SODIUM FLUORIDE-STIMULATED LUTEAL ADENYLATE CYCLASE BY CA^{+2}.

Sodium Fluoride (NaF) is a potent stimulator of mammalian adenylate cyclase. Concentrations of NaF of 5mM or greater was reported to produce maximal activation of adenylate cyclase in most mammalian adenylate cyclases studied(110). In the present studies, a concentration of 10mM NaF caused an eight fold or greater increase in enzyme

activity than that seen with LH(1 ug/ml) stimulation alone (Figure 9 and Table 5). NaF activation of luteal adenylate cyclase is independent of LH stimulation, 10mM NaF produced the same effect on enzyme activity in the presence or absence of LH (Table 5). Ca^{+2} produced a dose-dependent inhibition of NaF-stimulated adenylate cyclase activity. The half maximal(ID_{50}) of Ca^{+2} inhibition of NaF was about 100uM whereas the ID_{50} of the Ca^{+2} inhibition of LH-stimulated enzyme activity was 10 fold less. Addition of 10uM GppNHp, but not of 100uM GTP, decreased the magnitude of NaF stimulation and this decrease was more pronounced in the absence of LH (Table 5).

IV.6 EFFECT OF CA^{+2} ON THE BINDING OF GTP OR GPPNHP AND ON RELEASE OF GPPNHP FROM LUTEAL MEMBRANES.

The dissociation of GDP or the binding of GTP has been suggested to be the rate limiting steps in hormone stimulation of cAMP formation. Ca^{+2} concentrations greater than 5uM attenuated irreversibly the ability of GTP or GppNHp to stimulate LH dependent adenylate enzyme in luteal membrane preparation (results not shown). Addition of 20uM Ca^{+2} to the enzyme led to a 25-50% decrease in LH dependent cyclase activity even in the presence of high GTP concentrations and a greater degree of inhibition was seen

even in the presence of GppNHp (Figure 6 and 7). On the other hand, inhibition by 1uM Ca^{+2} was reversed with 1-10uM GTP or 0.1uM of GppNHp. Based on the above results, several attempts were made to examine whether Ca^{+2} may interfere with binding of guanine nucleotides to or the dissociation of guanine nucleotides from the adenylate cyclase.

Results from experiments in which the effect of LH and/or Ca^{+2} on the binding of ^{32}P-GTP to adenylate cyclase in luteal membrane preparation was examined and are shown in Figure 10a-e. AppNHp was included in the reaction to inhibit nucleotide hydrolysis which was reported to be minimal when used under similar condition(59, 107). The amount of radioactivity bound after incubation of ^{32}P-GTP with luteal membranes increased with time and decreased by raising the concentrations of cold GTP in the reaction mixture (Figure 11). Because ^{32}P was used in this experiment, it was not possible to directly measure the amount of cAMP formed by radioimmunoassay(see Materials and Methods).

In figure 10a, during the period of maximal cyclase activity, there was essentially no difference in the amount of radioactivity material bound under the conditions examined by after 10 of incubation. Figure 10b shows that the amount of radioactivity materials bound in the presence or absence of LH stimulation only; there were only small

differences in the binding of ^{32}P up to 10 minutes of incubation, but at intervals between 30 and 60 minutes, there was considerable higher counts in samples without LH. This may be consistent with hormone stimulation of GDP dissociation since the total amount of ^{32}P-GTP in each samples would be expected to decrease gradually over time despite a nucleotide regeneration system used in the assay (See Materials and Methods). On the other hand, as shown in Figure 10d, in the presence of 20uM Ca^{+2}, this difference between the presence or absence of LH was not observed; the amount of radioactivity from ^{32}P-GTP that was bound was not altered by LH. Also, the presence or absence of Ca^{+2} did not consistently affect the amount of radioactivity bound to membranes and this was seen independent of LH stimulation (Figure 8c, 8e). Either Ca^{+2} does not inhibit LH-dependent luteal adenylate cyclase by interfering with binding or dissociation of guanine nucleotides, or the sensitivity of the binding assay may not be adequate to detect the changes in binding of guanine nucleotides by Ca^{+2}. Also, since basal adenylate cyclase activity was always several folds lower than LH-stimulated enzyme activity, figure 10b also shows that the amount of guanine nucleotides bound did not correspond to the degree of enzyme activity.

Attempts were also made to examine whether Ca^{+2} affected

the binding of ^{3}H-GppNHp to adenylate cyclase in luteal membrane preparations. Filter binding studies with Ca^{+2} and ^{3}H-GppNHp did not show any differences over time intervals examined (results not shown). Not only was the amounts of ^{3}H-GppNHp bound not changed overtime, but the binding of ^{3}H-GppNHp was essentially at maximum within 1 minute at 30C, and therefore the radioactivity bound, like the results from the GTP binding studies, did not correlate with the enzyme activity. Another approach was used based on the GppNHp binding studies in turkey erythrocytes by Cassel and Selinger(109), where instead of filtration, a wash and centrifugation was used(see materials and methods); these results are shown in Table 6. The amount of membrane bound ^{3}H-GppNHp increased with time with the maximum amount bound between 10-15 minutes. The amount of membrane bound ^{3}H-GppNHp in samples with 20uM Ca^{+2} or without 20uM Ca^{+2} were essentially the same between 5 to 30 minutes, but the rate of cAMP formation differed. The rate of cAMP formation was stable from 5 to 30 minutes of incubation at 30C. The enzyme activity was approximately 20-33% less in the presence of 20uM Ca^{+2} at 30C while the amount of ^{3}H-GppNHp bound was essentially similar in the presence or absence of 20uM Ca^{+2}. The addition of 10^{-5}M unlabeled GppNHp to

^{3}H-GppNHp led to an approximately 8 fold increase in total GppNHp bound and a concomitant decrease in the amount of membrane bound radioactivity; the GppNHp binding was not saturated with 10^{-6}M GppNHp in a reaction mixture containing 1 mg/ml of membrane protein. At similar concentrations of GTP, 10^{-5}M to 10^{-6}M, a greater amount of guanine nucleotides was bound to membrane protein when tested with GppNHp than with GTP. We did not take into consideration any differences in the degree of nonspecific binding between ^{32}P-GTP or ^{3}H-GppNHp.

Since the amount of GTP or GppNHp bound to membrane protein did not appear to differ significantly in the presence or absence of 20uM Ca^{+2}, while the rate of cAMP formation was attenuated by the presence of Ca^{+2}, an attempt was made to evaluate the affect of 20uM Ca^{+2} on the exchange of guanine nucleotides. Luteal membranes bound with ^{3}H-GppNHp were incubated in the presence and absence of 20uM Ca^{+2} in a reaction mixture containing 10 ug/ml LH and an excess of unlabeled GTP in order to release labeled nucleotides from hormone-dependent sites at 30C (see Materials and Methods for releasing medium). The amount of ^{3}H-GppNHp released at different time intervals was measured and the results are shown in Figure 12. In the presence of

20uM Ca^{+2}, less ^{3}H-GppNHp was released during the time intervals measured. Since the releasing medium was similar to reaction mixture used to measure adenylate cyclase activity, cAMP formation was measured at the same time release of ^{3}H-GppNHp was measured and it was correlated with the amount of ^{3}H-GppNHp released. More ^{3}H-GppNHp was released in the absence of 20uM Ca^{+2} and this also corresponded to a higher enzyme activity. Ca^{+2} appeared to decrease the rate of exchange of ^{3}H-GppNHp with cold GTP and this was correlated with a lower adenylate cyclase activity.

IV.7 EFFECT OF MAGNESIUM CONCENTRATIONS ON CA^{+2}-INHIBITION OF LH-STIMULATED LUTEAL ADENYLATE CYCLASE.

The role of Mg^{+2} in Ca^{+2}-inhibition of LH-stimulated adenylate cyclase was examined and the results are shown in Figure 13. The results are based on total Mg^{+2} concentrations. Mg^{+2} was required for activation of enzyme activity, there was no measurable activity in the absence of Mg^{+2} and less than basal enzyme activity with 1mM Mg^{+2}. Enzyme activity increased with increasing total concentrations of Mg^{+2} with maximal activity at 5mM Mg^{+2}. Adenylate cyclase activity was decreased between Mg^{+2}

concentrations of 5mM to 10mM and enzyme activity was further reduced with higher Mg^{+2} concentrations. For example, a Mg^{+2} concentration of 30mM yielded only 19% of maximal activity seen with 5mM Mg^{+2} in the absence of Ca^{+2}. Ca^{+2} inhibited adenylate cyclase activity in a dose-dependent manner in the presence of Mg^{+2}. This effect was clearly seen in the presence of 3mM and 5mM Mg^{+2}.

The degree of inhibition by 20uM Ca^{+2} was 100%, 78%, 44%, 19% and 12% with presence of 1mM, 3mM, 5mM, 10mM, 30mM Mg^{+2} respectively (Figure 14). When the concentration of Mg^{+2} was increased, the relative % inhibition by Ca^{+2} was decreased; this was true in the range of Ca^{+2} concentrations tested. Also, increasing the concentrations of Mg^{+2} reduced the inhibition of adenylate cyclase by Ca^{+2}. Thus, although high concentrations of Mg^{+2} was inhibitory, it also attenuated the inhibitory effect of Ca^{+2} on luteal adenylate cyclase.

IV.7 EFFECT OF MANGANESE CONCENTRATIONS ON CA^{+2}-INHIBITION OF LH-STIMULATED ADENYLATE CYCLASE.

In the absence of Mg^{+2} or Mn^{+2}, there was no measurable luteal adenylate cyclase activity (Figure 13 and 15). The

results are expressed in terms of total Mn^{+2} concentration. Mn^{+2} apparently could substitute for Mg^{+2} for activation of luteal adenylate cyclase. In the absence of Ca^{+2}, 5mM Mn^{+2} stimulated adenylate cyclase 2-3 fold more than 5mM of Mg^{+2}. Maximal enzyme activity was seen with Mn^{+2} concentrations between 5-10mM but higher concentrations of Mn^{+2} were inhibitory; this was similar to that seen with Mg^{+2}. More significantly, while Ca^{+2} inhibited Mg^{+2} dependent adenylate cyclase in a dose dependent manner, Ca^{+2} had no effect on Mn^{+2}-activated enzyme activity (Figure 16); Mn^{+2} also increased the activity of adenylate cyclase independent of Ca^{+2} concentration. Concentrations of Ca^{+2} between 10-100uM inhibited Mg^{+2} dependent adenylate cyclase activity from 49 to 71% respectively, in the presence of 5mM Mg^{+2}, but the addition of 5mM Mn^{+2} eliminated this inhibition and even increased the enzyme activity to approximately twice that seen with 5mM Mg^{+2} in the absence of Ca^{+2} inhibition.

In addition, the effect of Mn^{+2} on adenylate cyclase activity was independent of LH stimulation, since enzyme activity was the same in the presence or absence of LH (Table 7). Since Mn^{+2} can activate luteal adenylate cyclase

in the absence of LH, this would implied that mechanisms of Mn^{+2} stimulation of adenylate cyclase occur independently of interaction of hormone receptors with other adenylate cyclase components. The result of Mn^{+2} stimulation was greater than that of maximal LH stimulation, but the effect were not additive.

DISCUSSION

Recent work(96) from our laboratory indicated that Ca^{+2} is an important intracellular ion in the regulation of luteal adenylate cyclase activity and may be the intracellular mediator of natural luteolytic agents such as PGF2a and LHRH. In vitro studies by Behrman et al.(41) showed that increasing intracellular Ca^{+2} leads to a decrease in LH-stimulated cAMP formation and progesterone secretion identical to that seen with PGF2a and LHRH in intact luteal cells. The present studies show a direct inhibition by Ca^{+2} on cAMP accumulation in purified plasma membranes of the rat corpus luteum which is in agreement with earlier reports by Behrman et al.(41, 96).

Inhibition of adenylate cyclase activity by Ca^{+2} is based on a direct assay of the membrane-bound enzyme activity in the presence of Ca^{+2}. Ca^{+2}-inhibition of adenylate cyclase activity was rapidly reversible when the concentration of Ca^{+2} is reduced by addition of EGTA to the reaction mixture. For example, addition of 5mM EGTA, after preincubation of membrane-bound enzyme with Ca^{+2}, led to a complete reversal of the inhibition of LH-sensitive adenylate cyclase activity by Ca^{+2} (Table 5). EGTA also

reversed Ca^{+2}- inhibition of basal adenylate cyclase activity(96).

Previous studies from Behrman et al. showed that Ca^{+2} in the assay did not decrease substrate levels of ATP or increase cAMP degradation(41). However, Ca^{+2} did not inhibit enzyme activity by an action on the LH receptor since Ca^{+2} did not affect the affinity or the binding capacity of the LH receptor for its ligand(41). Moreover, Ca^{+2} also inhibited basal adenylate cyclase activity. Ca^{+2}, in the concentrations used in the current studies, would therefore not be expected alter substrate ATP concentrations in the assay(96). Recently, Behrman et al.(96) also showed that Ca^{+2} over a range of concentrations had no significant effect on GTPase activity and that LH, PGF2a, or LHRH also had no significant effect on GTPase activity. They(96) concluded that Ca^{+2}-inhibition of adenylate cyclase did not result from an increase in the rate of GTP degradation by the guanine nucleotide regulatory protein(G) of luteal adenylate cyclase.

The current studies show a dose-dependent inhibition of luteal adenylate cyclase in membrane preparations by Ca^{+2} in the concentration range of luM to as high as 800uM (Table 5 & Figure 4). Half-maximal inhibitory concentration of Ca^{+2}

occurred between 10 to 20uM for LH-stimulated enzyme activity. Our laboratory has reported earlier that significant inhibition of luteal adenylate cyclase by Ca^{+2} could be achieved with concentrations as low as 0.5-1uM. Behrman et al.(96) reported earlier that the sensitivity of adenylate cyclase in luteal plasma membranes to inhibition by Ca^{+2} was increased by several fold with preincubation of membranes with Ca^{+2} for 20-60 min at 4C or 5 min at 37C(96). Free cytosolic Ca^{+2} concentrations in most cells at rest varies between 0.05 to 0.5uM(44), but the Ca^{+2} concentration may rise rapidly to the range of 1 to 2uM(44) or higher(95) when stimulated. Also in many cells, a nonuniform distribution of Ca^{+2} is often seen which could result in a much higher level of Ca^{+2} in localized areas of the cytoplasm(44).

At present, no direct information is available on role of Ca^{+2} in corpus luteum function or the reproductive cycle. A large increase in Ca^{+2} concentration was found in cumulus-enclosed oocytes of rats after injection of PMSG with maximal concentration of Ca^{+2} approximately 55h later, when ovulation occurs (112). It is known that the corpus luteum secretes progesterone under the influence of LH by activating the membrane-bound adenylate cyclase. In view of

the fact that a large change in intracellular Ca^{+2} is found to take place in cells of the corpus luteum(112), that a Ca^{+2} concentration of 1 to 2uM Ca^{+2} which is consistent with cellular physiology could lead to significant inhibition of membrane-bound luteal adenylate cyclase activity, and that this inhibition of the enzyme activity by Ca^{+2} could be rapidly reversed, strongly implies that Ca^{+2} is an important intracellular messenger in the luteal cell.

It is currently understood that adenylate cyclase can be regulated by a pair of homologous guanine-nucleotide-binding regulatory proteins--a G_s that mediates stimulation of adenylate cyclase activity, and a G_i that is responsible for inhibition. Although a fair amount is known about the structural similarities between G_s and G_i and their similar requirements for activation, the mechanism of their interaction in regulating the activity of the catalytic component of the system is still unclear. There is no concrete evidence that rat luteal adenylate cyclase contains both a G_s and G_i regulatory site, but there is indirect evidence for the existence of both a G_s and a G_i component in the rabbit luteal adenylate cyclase(65). Therefore interpretation of the possible modes of Ca^{+2} interaction with rat luteal adenylate cyclase would need to take into

account the existence of G_s and G_i.

Ca^{+2}-inhibition of adenylate cyclase is not only reversed by EGTA alone, it is also reversed by GTP and GppNHp, but only when low level of Ca^{+2} are used (<2.5uM) and in the presence of LH stimulation. GTP and GppNHp in concentrations of 10uM and 0.1uM respectively, could reverse inhibition of adenylate cyclase by 1uM of Ca^{+2} (Figure 6 & 7), but only GppNHp at concentrations of 100uM or greater could reverse inhibition of the enzyme by 2.5uM of Ca^{+2}(results not shown). Inhibition by Ca^{+2} at concentrations greater than 2.5uM, was not prevented by high concentrations of GTP nor by GppNHp (Figure 6 & 7). Ca^{+2}-inhibition of basal adenylate cyclase activity was not reversed by GTP or GppNHp (Figure 8). This observation suggests that GTP-blockade of inhibition by low concentrations of Ca^{+2} is a hormone dependent process.

In addition, results from current work and others(59, 109) have suggested that hormone stimulation of the membrane-bound enzyme leads to an increase in the exchange of guanine nucleotides. Earlier results also showed that neither Ca^{+2} or LH have any significant effect on GTPase activity in luteal adenylate cyclase(96). Therefore, it appears that under conditions of LH stimulation, GTP or

GppNHp is able to interact in a competitive manner with low concentrations of Ca^{+2} and in a non-competitive manner with higher concentrations of Ca^{+2} (at approximately >2.5uM) to alter the activity of luteal adenylate cyclase. Although the site of interaction of Ca^{+2} with the subunit(s) of adenylate cyclase is unknown, our results suggest that Ca^{+2} may interfere with LH-induced exchange of guanine nucleotides, a process that is necessary for activation of adenylate cyclase in a variety of cell types(61). But since both G_s and G_i of the adenylate cyclase regulatory subunits are also GTPases(114), it is unclear on which subunit that Ca^{+2} effects guanine nucleotides exchange that would consequently lead to inhibition of adenylate cyclase.

Fluoride-stimulated adenylate cyclase activity is known to be independent of hormone(LH) activation. This is consistent with the original work by Sutherland et al.(113). Fluoride-stimulated enzyme activity in luteal membrane preparations was inhibited by Ca^{+2} in a dose-dependent manner although a higher concentration of Ca^{+2}, approximately 100uM or greater, was required to achieve half-maximal inhibition relative to that seen with inhibition of LH-stimulated adenylate cyclase by Ca^{+2} (Table 5). In contrast to earlier work of Sutherland et al.(113),

who suggested that fluoride acts directly on the catalytic unit of the enzyme, Aurbach et al.(107) showed that the guanine nucleotide regulatory protein is necessary for fluoride activation of adenylate cyclase and that the nature of the guanine nucleotide on the regulatory site influences fluoride stimulation. Aurbach et al.(107) group also showed that exchange of the guanine nucleotides on the regulatory site with other nucleotides in the incubation medium is not necessary for fluoride stimulation of enzyme activity; endogenous GDP, tightly bound to the guanine nucleotide regulatory protein, is sufficient for supporting fluoride stimulation of adenylate cyclase activity(107). The ability of Ca^{+2} to affect fluoride-stimulated enzyme activity may indicate that Ca^{+2} interacts at the regulatory subunit but this does not exclude the possibility that Ca^{+2} may also interact at other sites. Recent work from Birnbaumer et al.(114) and others showed that fluoride inhibits the GTPase activity of G_i while it also activates G_i and thus leads to an inhibition of adenylate cyclase activity in purified G_i from CYC^- cells. Fluoride-activated adenylate cyclase activity presented here is reflective of its stimulative affect on G_s since increasing concentrations of fluoride leads to a rise in adenylate cyclase activity in the absence of Ca^{+2}. The membrane-bound luteal enzyme used in the

current studies appears to contain both a G_s and G_i regulatory site since fluoride-activated adenylate cyclase was inhibited by stable guanine nucleotides; this effect of stable guanine nucleotides on fluoride-activated adenylate cyclase has been shown in rabbit luteal cyclase(65) and in CYC^- cells(114). The nature of Ca^{+2}-inhibition of fluoride-activated luteal adenylate cyclase appears to differ from that of the LH-stimulated enzyme since a significantly higher concentration of Ca^{+2} (100uM or greater) was required for an equivalent degree of inhibition. In addition, low levels of Ca^{+2} (~2.5uM) inhibited LH-stimulated adenylate cyclase activity by ~40%, whereas this level of Ca^{+2} did not inhibit fluoride-stimulated enzyme activity. It has been reported(66, 115) that fluoride alone could activate G_s or G_i, and if Ca^{+2} could enhance fluoride's inhibition of G_i, we suggest that Ca^{+2} acts either at G_i or directly inhibits the activity of the catalytic subunit.

Forskolin, a plant diterpene, is a potent activator of adenylate cyclase from virtually all mammalian cells and tissues, as well as in broken cell and solublized preparations(116). It is also a potent activator of rat luteal adenylate cyclase(96) independent of LH stimulation.

Early work showed that forskolin activates the catalytic subunit of adenylate cyclase directly without the presence of the G_s but with an intact G_i in CYC^- cells, and therefore, it suggested that the compound acts at a site on the catalytic subunit or a closely associated protein(116). However, recent studies(117-120) showed a striking interdependence of forskolin and receptor-mediated G_s input in the activation of adenylate cyclase in many agonist systems; the presence of hormone-activated G_s potentiated the effect of forskolin on cAMP-production in intact cells(117). Moreover, the stimulation of cAMP-production by forskolin in intact cells is found to be inhibited by receptor-mediated G_i(117). Also, several studies recently showed that forskolin-stimulation of adenylate cyclase in CYC^- cells(122, 121) and in rabbit luteal cell membranes(65) can be inhibited by nonhydrolysable guanine nucleotide analogs on G_i. Ca^{+2} ions are found to inhibit forskolin responses in both intact cells and membranes(116, 118, 123), but only when high concentrations in the millimolar range are used. In contrast to earlier work by others(116, 118, 123), our recent results(96) showed that forskolin-activated luteal adenylate cyclase is extremely sensitive to Ca^{+2} inhibition; 5uM Ca^{+2} led to a greater than 50% inhibition of

the enzyme activity stimulated with 100uM forskolin. The concentration of Ca^{+2} required to achieve half-maximal inhibition of forskolin-activated luteal adenylate cyclase is significantly lower than that for fluoride-activated enzyme activity seen in the current study, but is similar to that for LH-stimulated enzyme activity. Based on our result which showed that Ca^{+2} acutely inhibited forskolin-activated adenylate cyclase, and others(65, 122, 121) which showed that forskolin-activated adenylate cyclase could occur in the absence of G_s or hormone stimulation, it would indicate that Ca^{+2} may activate G_i or directly inhibit the catalytic subunit. Both of the action of Ca^{+2} would lead to inhibition of cAMP accumulation. Since inhibition of adenylate cyclase at low concentration of Ca^{+2} could be reversed by guanine nucleotides, it would be interesting to investigate whether guanine nucleotides would reverse Ca^{+2}-inhibition of forskolin-activated adenylate cyclase in the presence of LH. This could possibly argue for a site of action of Ca^{+2} on G_s; but this form of reversal could also indicate that hormone activated G_s is competitively inhibited by Ca^{+2}-activated G_i.

Studies which showed the effect of Mg^{+2} as a regulator of adenylate cyclase are numerous and have been recently

reviewed by Cech et al.(87). Aside from the fact that Mg^{+2} determines the relative stimulation that can be elicited at any given guanine nucleotide concentration with rabbit luteal cyclase, Birnbaumer et al.(53) recently suggested that Mg^{+2} in micromolar concentrations activates G_i and leads to a change in the conformation of G_i and the stimulation of G_i-dependent GTPase activity. However, Mg^{+2} in millimolar concentrations is required for activation of G_s which then lead to stimulation of adenylate cyclase(53). They(66) also showed with CYC^- cell membrane preparations that Mg^{+2} in the low micromolar range leads to a relative decrease in adenylate cyclase activity but only with the addition of guanine nucleotides; this decrease is relative to the control which has no added guanine nucleotides. However, the rate of cAMP formation continues to increase in the absence and presence of guanine nucleotides when the Mg^{+2} concentration is increased. Intracellular concentrations of free Mg^{+2} is in the range of 0.5-1.0mM(124) and many intracellular enzymes require Mg^{+2} at this concentration range for activation, it is therefore unlikely that intracellular concentrations of Mg^{+2} could be decreased to the low micromolar range to mediate hormone induced inhibition of adenylate cyclase. On the other hand,

these studies emphasize the important role for divalent cations in regulation of adenylate cyclase. Also, the intracellular concentration of GTP is in large excess in mammalian cells and would therefore be unlikely to have a regulatory role in the adenylate cyclase system(125).

The present studies show that Mg^{+2} is critical for activation of luteal adenylate cyclase, and a total concentration greater than lmM is required for expression of luteal adenylate cyclase activity. But even when hormone-stimulated luteal adenylate cyclase is fully activated in the presence of an optimum concentration of Mg^{+2}, it continues to be exquisitely sensitive to Ca^{+2} inhibition in micromolar concentrations that is consistent with physiological levels of Ca^{+2}. Ca^{+2} also inhibited adenylate cyclase activity at higher concentrations of Mg^{+2}, but higher concentrations of Mg^{+2} were also inhibitory on adenylate cyclase activity. High concentrations of Mg^{+2} has been shown to increase the affinity of gonadotropin binding only when measured after a 30-40 minutes of incubation(126); this increase was minimal with short incubations and therefore should not contribute to the inhibition of adenylate cyclase in our studies.

Similar to results found with rabbit luteal adenylate

cyclase(53), LH activation of the enzyme is optimal only over a narrow range of Mg^{+2} concentrations. The relative degree of Ca^{+2} inhibition of adenylate cyclase at higher concentrations of Mg^{+2} was attenuated. There appears to be competitive inhibition between Ca^{+2} and Mg^{+2} when assayed with higher concentration of Mg^{+2} but the kinetics of this inhibition require further exploration. It is clear from these studies that Mg^{+2} plays an important role in the activation of adenylate cyclase. Since the intracellular free Mg^{+2} concentration varies between 0.5-1mM and although fluctuation in Mg^{+2} concentrations has been noted in cells, micromolar concentrations of Mg^{+2} has never been recorded, whereas the concentrations of Ca^{+2} that inhibit luteal adenylate cyclase activity are well within the physiological range.

Mn^{+2}, on the other hand, at all concentrations tested, consistently prevented Ca^{+2}-inhibition of adenylate cyclase activity. In the absence of Mg^{+2}, Mn^{+2} could serve to activate rat luteal adenylate cyclase in a dose-dependent manner but a concentration greater than 1mM is required. High concentrations of Mn^{+2}(10mM or greater) inhibit luteal adenylate cyclase activity, a finding that is consistent

with earlier reports(87) which showed that increasing concentration of Mn^{+2} inhibits the V_{max} of S49 wild cells. The present studies show that Mn^{+2} activates luteal adenylate cyclase independent of LH stimulation. This is consistent with earlier results(87) which show that Mn^{+2} is a direct stimulator of the catalytic subunit. But recent evidence also suggests that Mn^{+2} may act to inhibit G_i activation(121), an effect which would produce a similar result with adenylate cyclase as does pertussis toxin in blocking inhibition by G_i. Mn^{+2} was shown to abolish the action of fluoride on a G_i purified preparation from CYC^- S49 cells and subsequently antagonized the inhibitory effect of G_i when recombined with an intact adenylate cyclase system(66). In the present studies, Ca^{+2} at all concentrations tested did not inhibit Mn^{+2}-activated adenylate cyclase. However, Ca^{+2} in millimolar concentrations has been shown to inhibit Mn^{+2}-activated CYC^- adenylate cyclase with enzyme kinetics suggestive of a competitive inhibition(123). The ability of Mn^{+2} to activate CYC^- adenylate cyclase and other mammalian adenylate cyclase independent of hormone stimulation, implies that the action of Mn^{+2} is independent of G_s.

Whether Mn^{+2} interacts with G_i, or the catalytic subunit directly, cannot be concluded from the current studies, but it is clear that the inhibition of adenylate cyclase by Ca^{+2} that approximated physiological concentrations was completely reversed in the presence of Mn^{+2}. Mn-ATP is the physiological substrate in some lower eukaryotes and prokaryotes(87), but not in mammalian adenylate cyclase studies, with the exception of CYC^- variant of S49 and naturally soluble adenylate cyclase in rat testes(87). Also, the intracellular concentration of Mn^{+2} in mammalian cells is negligible. Therefore, although Mn^{+2} has proven to be a useful tool to further our understanding of the regulation of adenylate cyclase, the physiological relevance of Mn^{+2} remains unclear.

All three divalent cations, Ca^{+2}, Mg^{+2}, and Mn^{+2} inhibited luteal adenylate cyclase when used at high concentrations, but only Ca^{+2} was inhibitory at concentrations that could approximate intracellular levels. Therefore the K_i for Ca^{+2} is several hundred fold lower than than of Mg^{+2} or Mn^{+2} and its effective concentration is more suitable for an intracellular regulator. A transient change in intracellular Ca^{+2} concentration when stimulated by hormone or neurotransmitters has been well documented and

recently reviewed(44). On the other hand, Mg^{+2} was shown to have specific and unique properties with regard to hormone activation of luteal adenylate cyclase, but since the intracellular variation of Mg^{+2} is usually between 0.5mM to 1mM(124), it is unlikely that varying Mg^{+2} concentrations alone could be the intracellular regulator of luteal adenylate cyclase.

Behrman et al. has shown that with Ca^{+2}-ionophore(114) and other agents(42) which increase intracellular Ca^{+2} have led to an acute inhibition of LH-dependent cAMP accumulation and steroidogenesis in intact cells, a response that mirrored the acute effect of PGF2a and LHRH. On the other hand, the acute effect of PGF2a or LHRH was independent of extracellular Ca^{+2}(42). If Ca^{+2} is the intracellular mediator of PGF2a and LHRH, then the rise in intracellular Ca^{+2} must be attained rapidly from intracellular sources and the reversal of this rise, ie. the return of Ca^{+2} concentration to basal level, must also be rapid since persistent elevation of Ca^{+2} is found to be toxic to cells(44). There are two relative large intracellular pools of nonionic calcium, the mitochondrial matrix and the endoplasmic reticulum, and these two pools are in rapid exchange with cytosolic pool of Ca^{+2}. Studies(44, 95) have

shown that both the mitochondria and the endoplasmic reticulum could serve as a source of Ca^{+2} for cellular activation. Thus, based on our studies, a release of intracellular Ca^{+2} from these sequestered sites would be suffice to inhibit luteal adenylate cyclase activity.

It is concluded that Ca^{+2} appears to inhibit luteal adenylate cyclase by two combined effects. Low concentrations (~<2.5uM) of Ca^{+2} acutely inhibited LH-stimulated , forskolin-stimulated, and basal adenylate cyclase activity, while significantly higher concentrations of Ca^{+2} are required to illicit a similar degree of inhibition on fluoride-activated enzyme activity. Also, only inhibition of luteal adenylate cyclase by low Ca^{+2} concentrations (~<2.5uM) was prevented by the addition of guanine nucleotides. These results indicate that Ca^{+2} may interact at two different sites on the adenylate cyclase complex, a high affinity site that is blocked by guanine nucleotides and fluoride, and a low affinity site that is blocked by Mn^{+2} stimulation but not by guanine nucleotides, fluoride, or forskolin. On the other hand, based on studies(122) that showed that activated G_s and G_i may interact in a noncompetitive manner and based on results from our current studies, it is conceivable that Ca^{+2}

(~<2.5) may activate G_i. Subsequently, the Ca^{+2}-activated G_i may interact with hormone activated G_s in a competitive manner when G_i is activated by a low Ca^{+2} concentration (~<2.5uM), but only a noncompetitive interaction occurs when activated by a higher Ca^{+2} concentration. Also, if Ca^{+2} activate G_i, then Mn^{+2}, shown to affect the catalytic subunit or inhibit G_i activation, would logically prevent the inhibition by Ca^{+2}. Furthermore, Mg^{+2}, in concentrations consistent with cellular levels, appears to modulate the degree of hormone stimulation and consequently the rate of cAMP formation. In summary, both Ca^{+2} and Mg^{+2} appear to be important divalent ions in the regulation of adenylate cyclase. It seems possible that by varying cellular Ca^{+2} in response to hormonal stimulation, then adenylate cyclase could alternate between an active or inactive state; and Mg^{+2} would then serve to modulate the magnitude of hormone-stimulated adenylate cyclase activity.

In conclusion, we have reinforced our previous observations that luteal adenylate cyclase is extremely sensitive to small increases of physiologically relevant Ca^{+2} concentrations. The possible interaction of Ca^{+2} with other divalent ions in the luteal adenylate cyclase system have been presented. Based on our current studies, several

modes of possible interaction of Ca^{+2} and luteal adenylate cyclase have been explored. Although at present, the mechanism of Ca^{+2} regulation of luteal adenylate cyclase remains speculative, we have shown that Ca^{+2} is an important intracellular ion in the regulation of luteal adenylate cyclase activity.

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TABLES

Table 1. ADENYLATE CYCLASE ACTIVITY OF LUTEAL MEMBRANE PREPARATION.

	Specific Activities [pMole cAMP/mg Protein/Min]	
	Basal	LH-stimulated
Light Membrane	9 ±1.3	35 ±3.8
Heavy Membrane	4 ±0.3	8 ±2.2
Microsomal-Enriched	16 ±1.7	70 ±8.2

See Materials and Methods for preparation of light, Heavy, Microsomal-Enriched membrane fractions. The reaction mixture contained LH (1ug/ml). Results are expressed as Mean±SE; N = 3 groups.

Table 2. RELATIONSHIP BETWEEN TOTAL AND FREE CALCIUM WITH A CALCIUM ELECTRODE.

Ca^{2+}_{total} mM	Ca^{2+}_{free} uM	$P[Ca^{2+}_{free}]$ $-\log[Ca^{2+}_{free}]$	mV
0	0	0	-138
0.2	0.057	7.25	-135
0.4	0.152	6.8	-118.7
0.5	0.227	6.64	-109.1
0.55	0.275	6.56	-105.7
0.59	0.33	6.47	-102.4
0.64	0.41	6.39	-98.8
0.69	0.5	6.3	-95.1
0.73	0.62	6.21	-91
0.78	0.79	6.1	-86.6
0.82	1.04	5.98	-81.4
0.87	1.45	5.84	-74.7
0.88	1.69	5.77	-71.2
0.9	2.01	5.7	-66.9
0.92	2.47	5.61	-61.7
0.94	3.13	5.5	-54.2
0.95	4.2	5.38	-44.1
0.97	6.08	5.22	-34.2
0.99	9.8	5.01	-27.1
1	17.3	4.76	-22.4
1.02	29	4.54	-19
1.04	43.4	4.36	-16.3
1.08	83	4.08	-11.2
1.12	124	3.91	-7.8
1.16	165	3.78	-5.1
1.2	205	3.69	-2.2

The constants used in the calculations were $K^{Ca-EGTA}$ = 4.4×10^6 M^{-1}. Buffer contained 25mM Tris, 1mM EGTA (pH 7.5). [See Materials and Methods for calculation of Ca^{+2}_{free}.]

Table 3. EFFECT OF MG+2 ON FREE CA+2.

Mg^{+2}_{total} (mM)	mV	Ca^{+2}_{free} (uM)	mV	Ca^{+2}_{free} (uM)
0	-90.0	0.8	-84.4	1.0
3	-66.0	2.4	-45.0	4.5
5	-63.0	2.6	-42.6	4.7
10	-59.0	3.2	-40.6	5.2
20	-53.5	3.6	-38.7	5.5
30	-51.0	3.7	-37.5	5.9
50	-50.5	3.8	-36.8	6.1

Free calcium was determined based on correlating the measurements from the Orion Ca^{+2}-sensitive electrode(mV) with the free Ca^{+2} standard curve in Figure 1.

Table 4. REVERSAL OF CA+2 INHIBITION OF LH-STIMULATED ADENYLATE CYCLASE BY EGTA.

TREATMENT			Ca+2 (uM)			
LH	EGTA(5mM)		0	10	20	100
+	−	Rate	49.1	17.5	28.5	22.7
		Std.Error ±	7	2.6	3.3	2.1
		Inhibition %		64	42	54
+	+	Rate	76	107	115	62.4
		Std.Error ±	9.2	22	48	1.2
		Inhibition %		−	−	18
		Reversal %		+	+	+
−	−	Rate	11.9	8.1	7.9	7.2
		Std.Error ±	0.7	0.6	0.8	0.8
		Inhibition %		32	34	39
−	+	Rate	14.2	9	14	11.6
		Std.Error ±	0.8	0.7	1	0.7
		Inhibition %		37	−	18
		Reversal %		+	+	+

Rate = pmole cAMP/mg protein/min. Rate ± Std. Error = Mean ± Standard error; N=3. EGTA (5mM) was added to samples after luteal membranes were incubated with Ca+2 at 4C in a reaction mixture for 20 min. LH = 1 ug/ml. % inhibition was relative to rate without added Ca+2. Concentration of Ca+2 was determined based on adenylate cyclase assay conditions (Materials and Methods).

Table 5. CA+2 INHIBITION OF FLUORIDE-STIMULATED LUTEAL ADENYLATE CYCLASE ACTIVITY.

TREATMENT NaF(10mM)	LH		Ca+2(uM) 0	2.5	20	100	300
+	+	Rate	288	301	177	134	89
		Std.Error+/-	33	13	7	8	8
		Inhibition (%)		-	38	53	69
+	-	Rate	288	337	186	128	119
		Std.Error+/-	26	40	26	12	9
		Inhibition (%)		-	36	55	59
-	+	Rate	35.6	21.8	17.2	11.7	7
		Std.Error+/-	2.6	2.6	1.7	1.7	0.1
		Inhibition (%)		39	52	67	80

			No Ca+2		
			0	GppNHp(10uM)	GTP(100uM)
+	+	Rate	288	233	273
		Std.Error+/-	33	5	16
		Inhibition (%)		19	-
+	-	Rate	288	190	307
		Std.Error+/-	26	6	27
		Inhibition (%)		34	-

Rate = pMole cAMP/mg Protein/min. Reaction mixture contained LH (1 ug/ml). GTP and GppNHp were freshly prepared. Each point is the mean ±standard errors of 3 replicates.

TABLE 6. 3H-GPPNHP BINDING IN LUTEAL MEMBRANE PREPARATION.

TREATMENT		TIME(MIN)					
		0	5	10	15	30	60
NO CA+2	CPM	21600	21168	27716	26259	25623	36213
+3H-GPPNHP	RATE	0	177	177	184	174	233
PMOLE EQUIV.	BOUND	1.08	1.06	1.39	1.31	1.28	1.81
+20uM CA+2	CPM	18140	20046	25680	27912	26389	14505
+3H-GPPNHP	RATE	0	146	121	135	119	64
PMOLE EQUIV.	BOUND	0.91	1	1.3	1.39	1.32	0.7
NO CA+2 +3H-GPPNHP	CPM	15675	14427	20253	21590	14743	13097
+ 10uM COLD GPPNHP	RATE	0	198	297	226	261	209
PMOLE EQUIV.	BOUND	8.6	7.9	11.1	11.9	8.1	7.2

Each assay contained 3H-GppNHp = 2.0 x 10^7 CPM/nmole, and 0.1 nmole was added per 0.1 ml assay volume. CPM represents counts per minute of membrane bound 3H-GppNHp on the filter. Rate represents the mean pmole cAMP/mg protein/min of two samples. The pmole equivalent bound was calculated by taking account of the amount of cold GppNHp in the reaction mixture.

TABLE 7. EFFECT OF MAGANESE ON MAGNESIUM-DEPENDENT LUTEAL ADENYLATE CYCLASE.

TREATMENT:		+LH	-LH
MG+2 ONLY	RATE	58	11.9
	STD. ERROR +/-	6.4	0.7
MG+2 & MN+2	RATE	76.3	84
	STD. ERROR +/-	3	3.4

Concentration of Mg+2 was 5 mM (See Materials & Methods). Concentration of Mn+2 was 5 mM. LH concentraion was 1 ug/ml. Rate represents pMole cAMP/Mg Protein/Min and is the mean of three replicates.

FIGURES

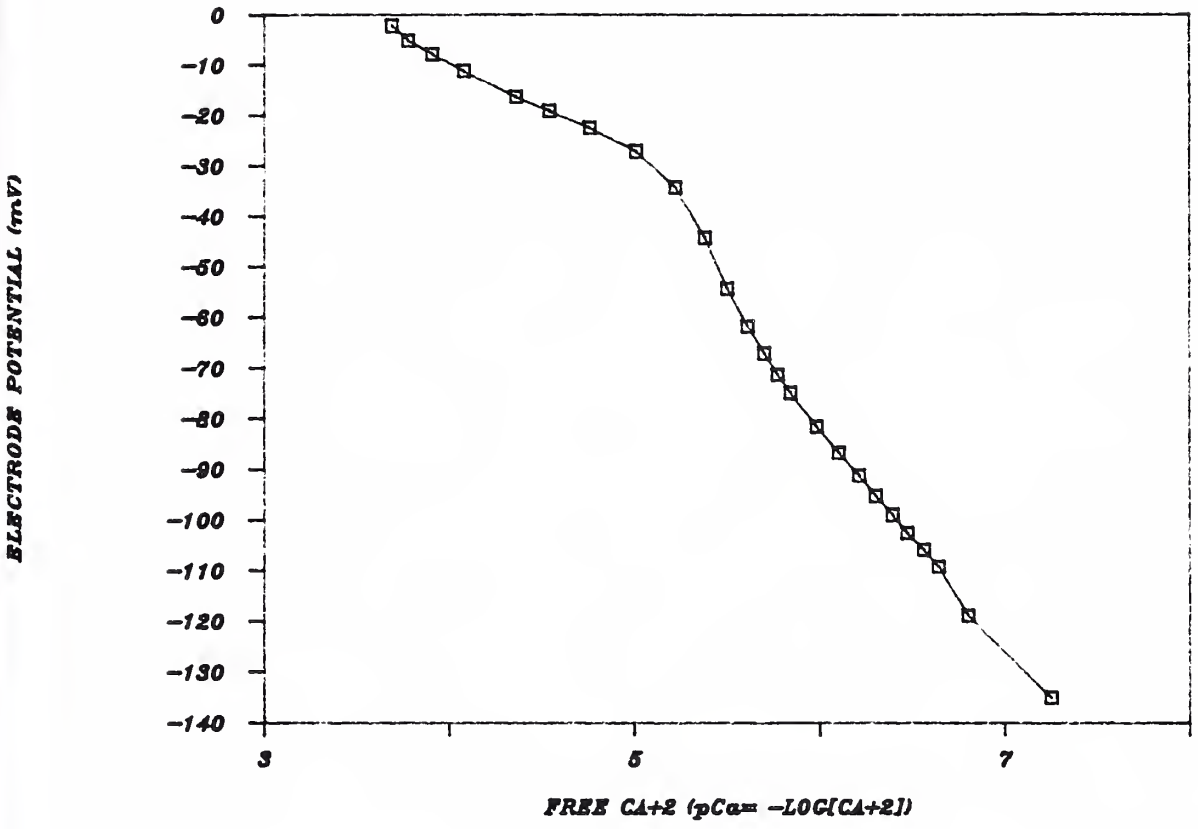

Figure 1. Free Ca^{+2} standard curve.

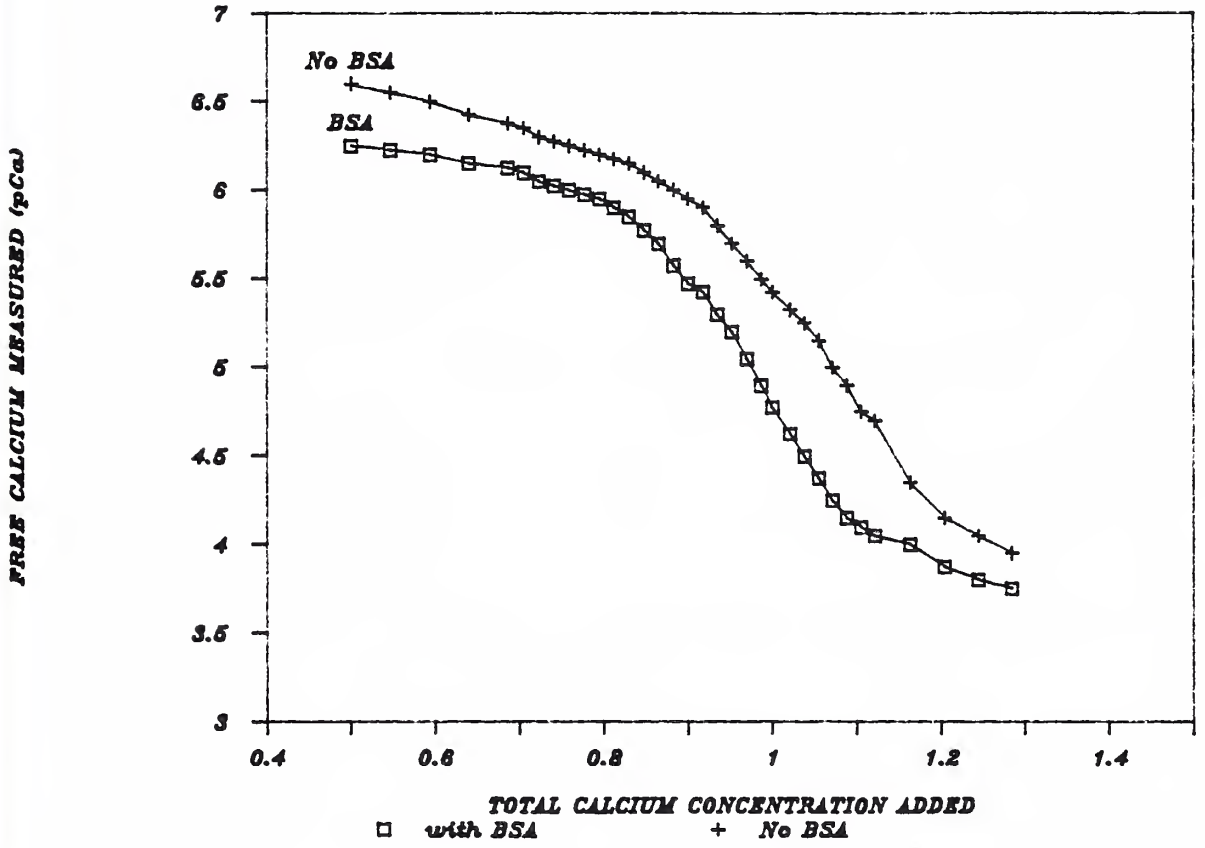

Figure 2. Free Ca^{+2} vs. Total Ca^{+2} in the presence and absence of BSA.

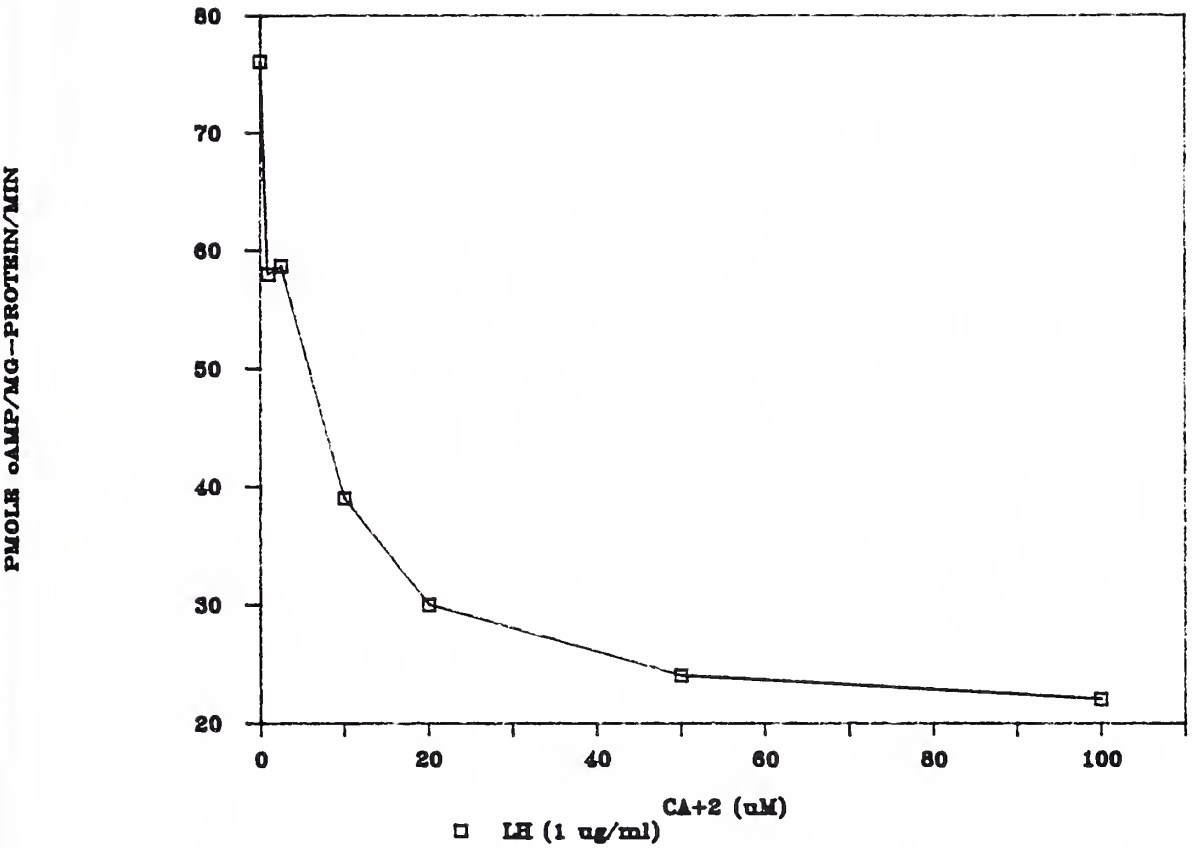

Figure 3. Inhibition of LH-stimulated adenylate cyclase activity by Ca^{+2}.

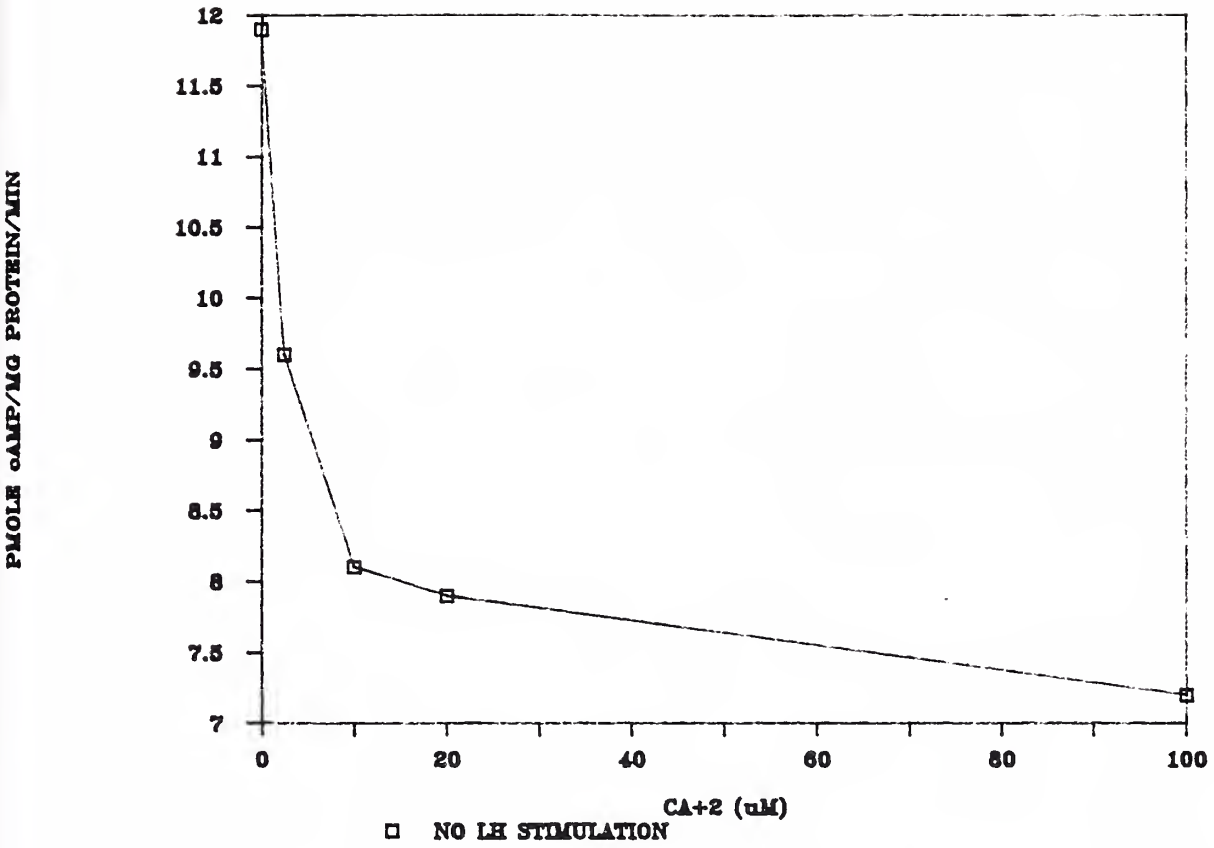

Figure 4. Inhibition of basal luteal adenylate cyclase activity by Ca^{+2}.

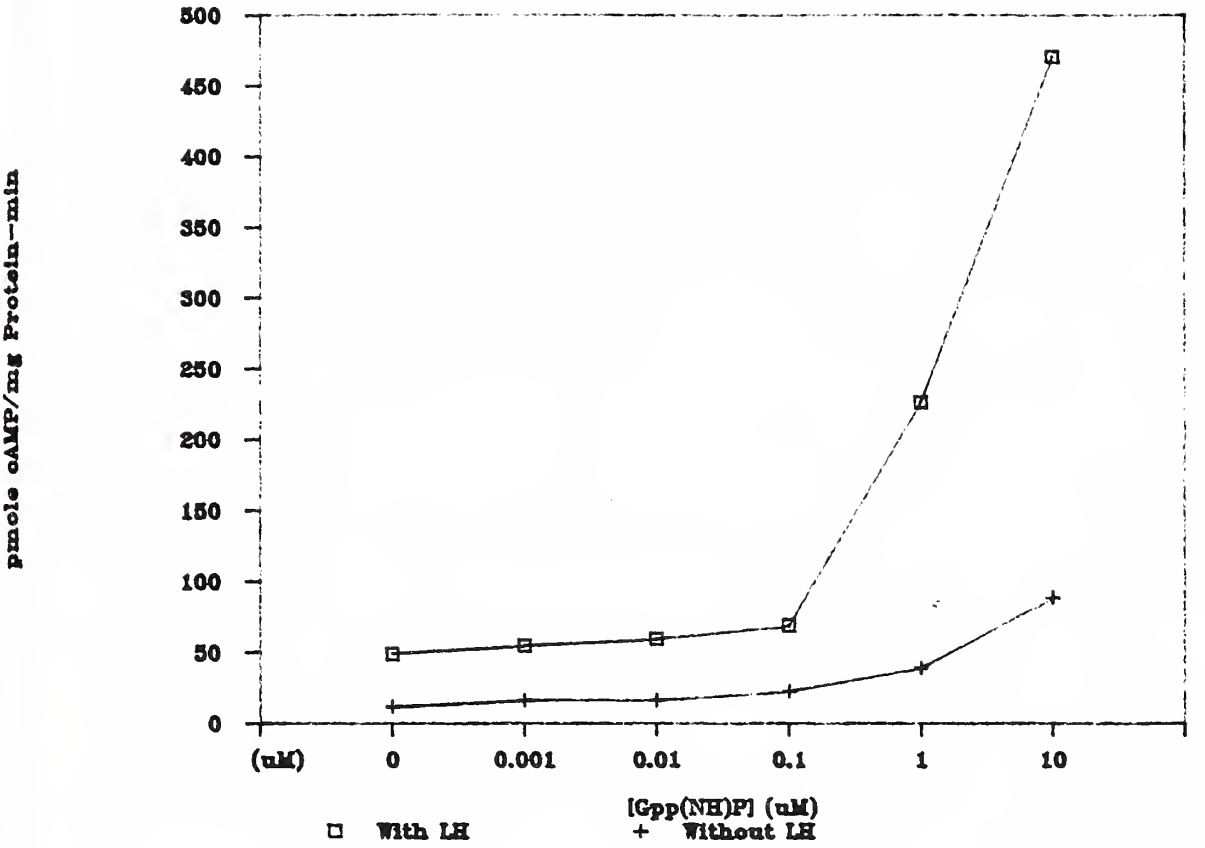

Figure 5. Effect of GppNHp on adenylate cyclase activity.

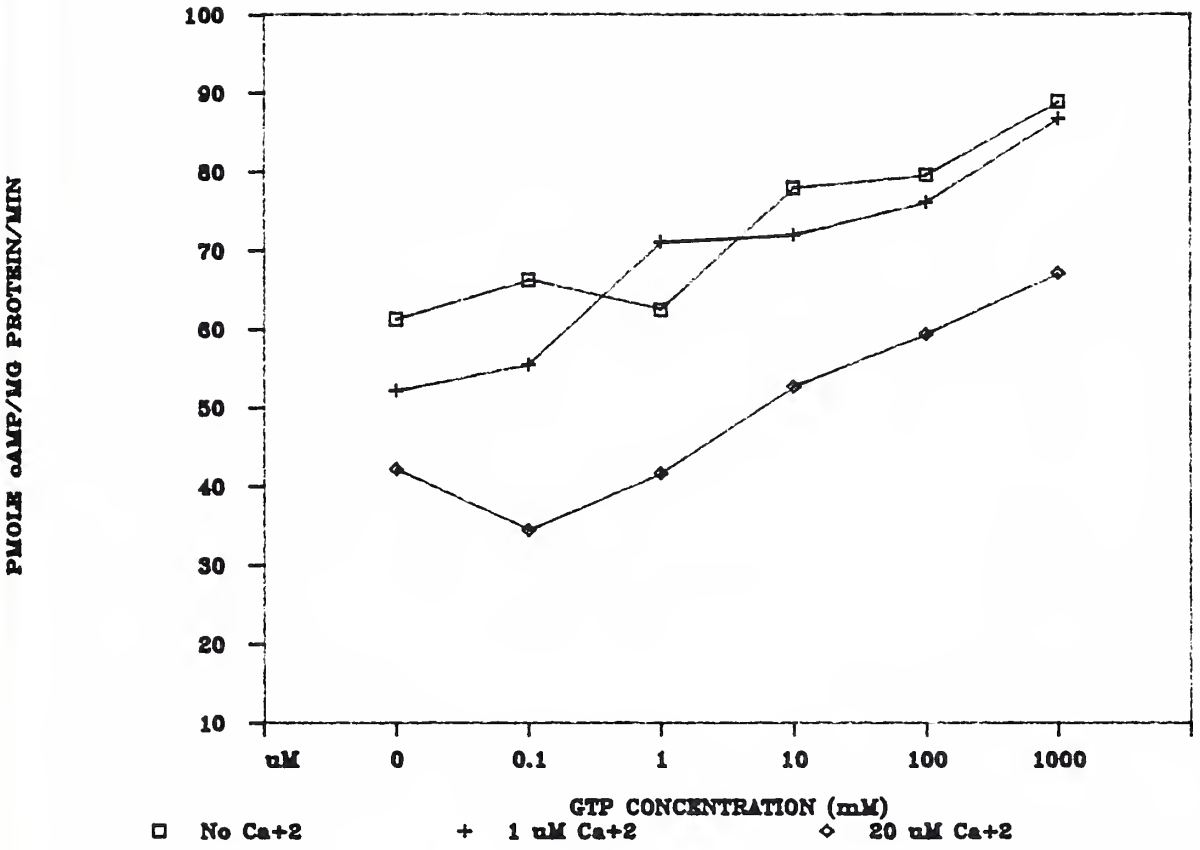

Figure 6. Effect of GTP on Ca^{+2}-inhibition of adenylate cyclase activity.

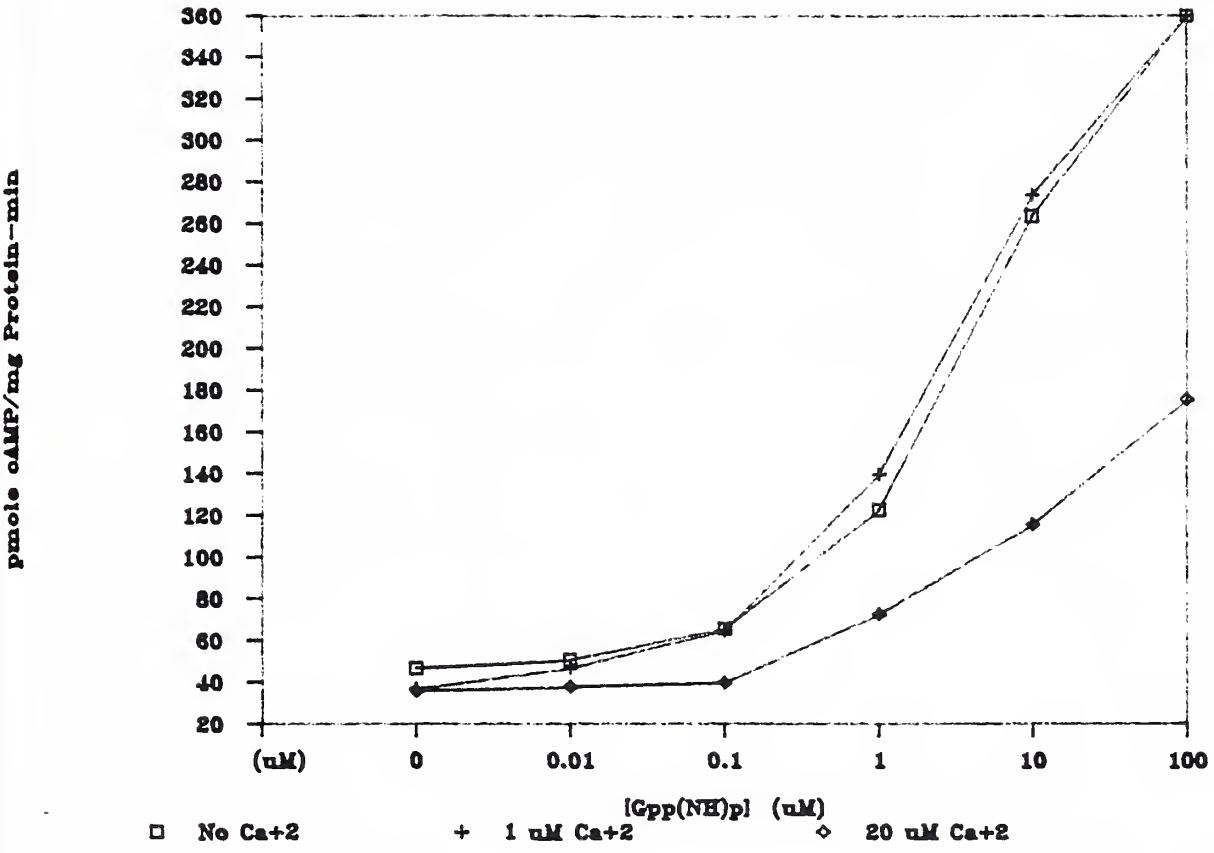

Figure 7. Effect of GppNHp on Ca^{+2}-inhibition of LH-stimulated adenylate cyclase activity.

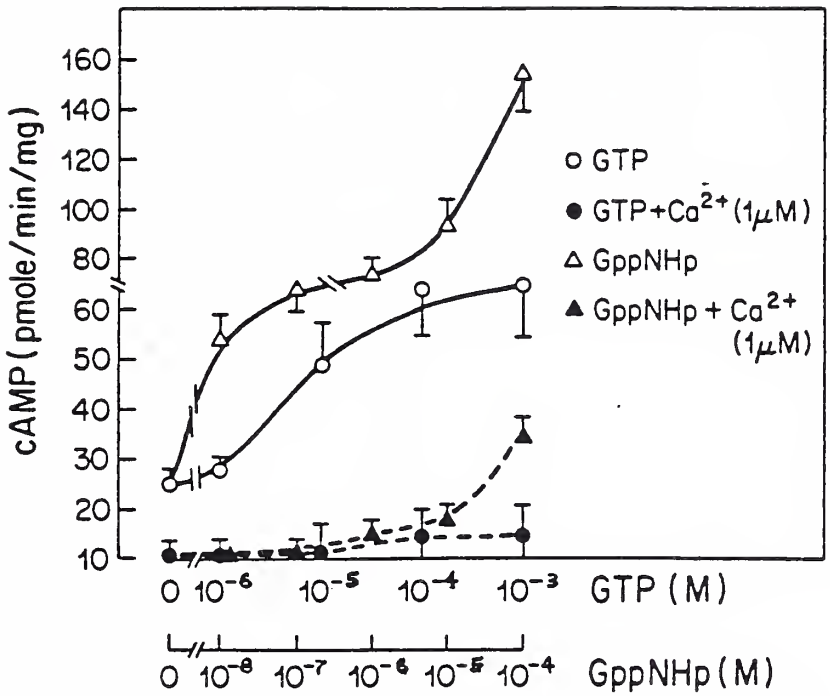

Figure 8. Effect of GTP and GppNHp on Ca^{+2}-inhibition of adenylate cyclase activity in the absence of LH.

(Copied with permission from H.R. Behrman.)

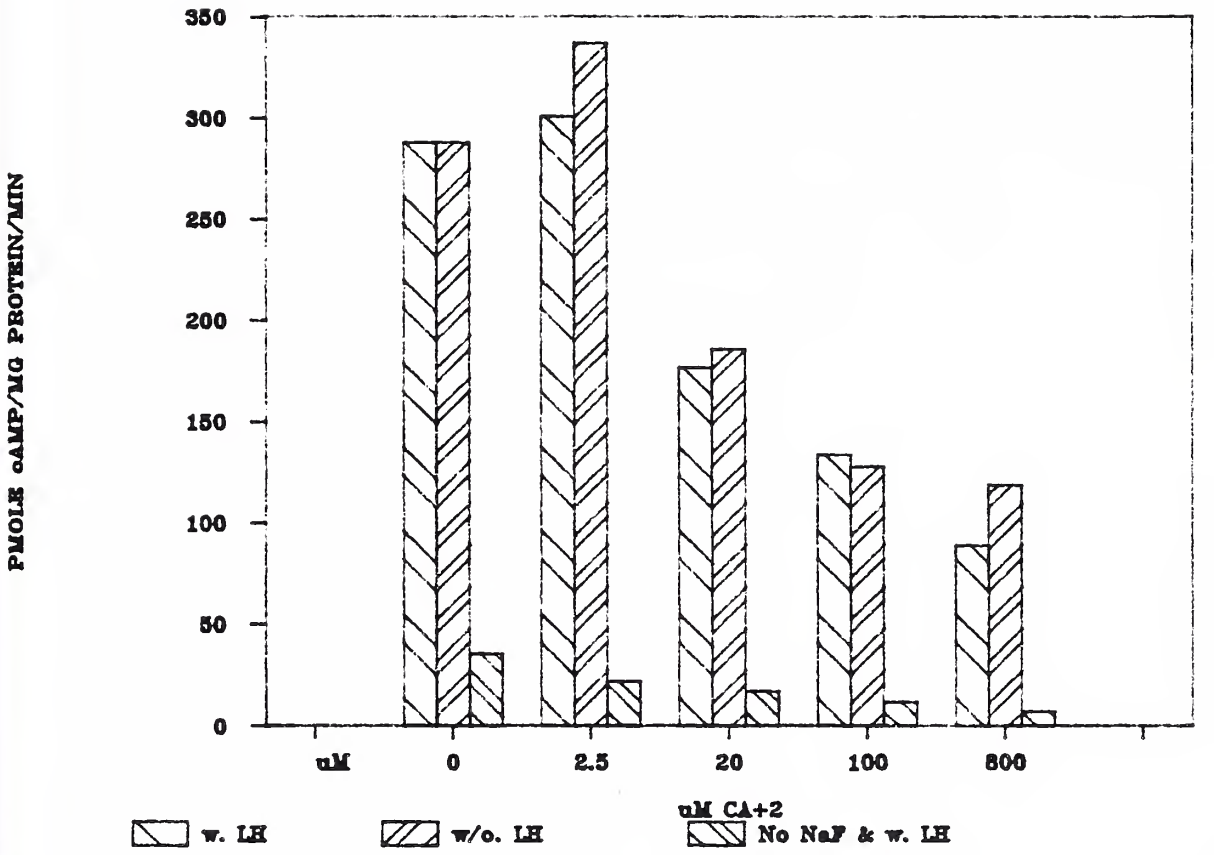

Figure 9. Effect of Ca^{+2}-inhibition of fluoride-stimulated luteal adenylate cyclase activity.

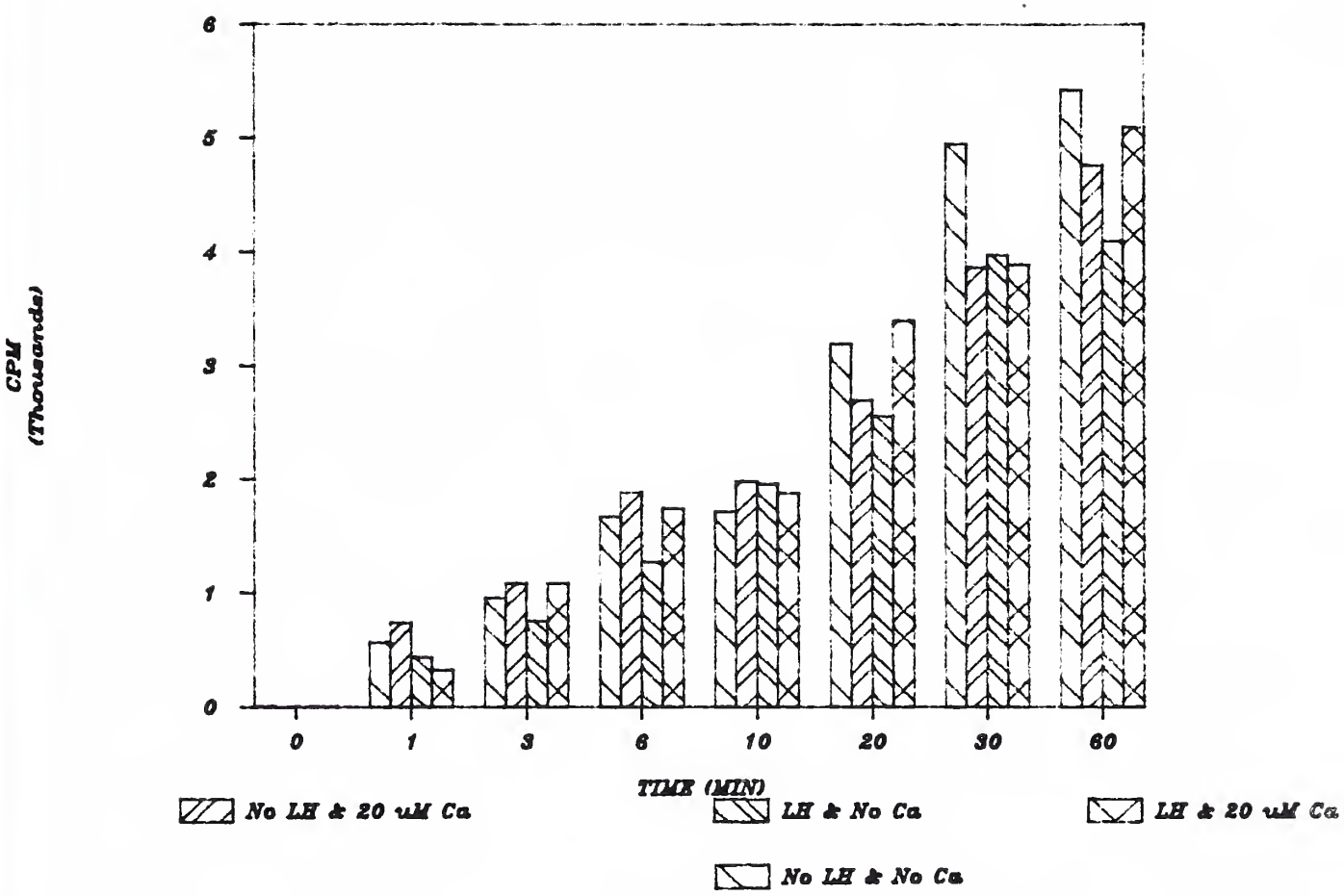

Figure 10a. Binding of ^{32}P-GTP to luteal membranes.

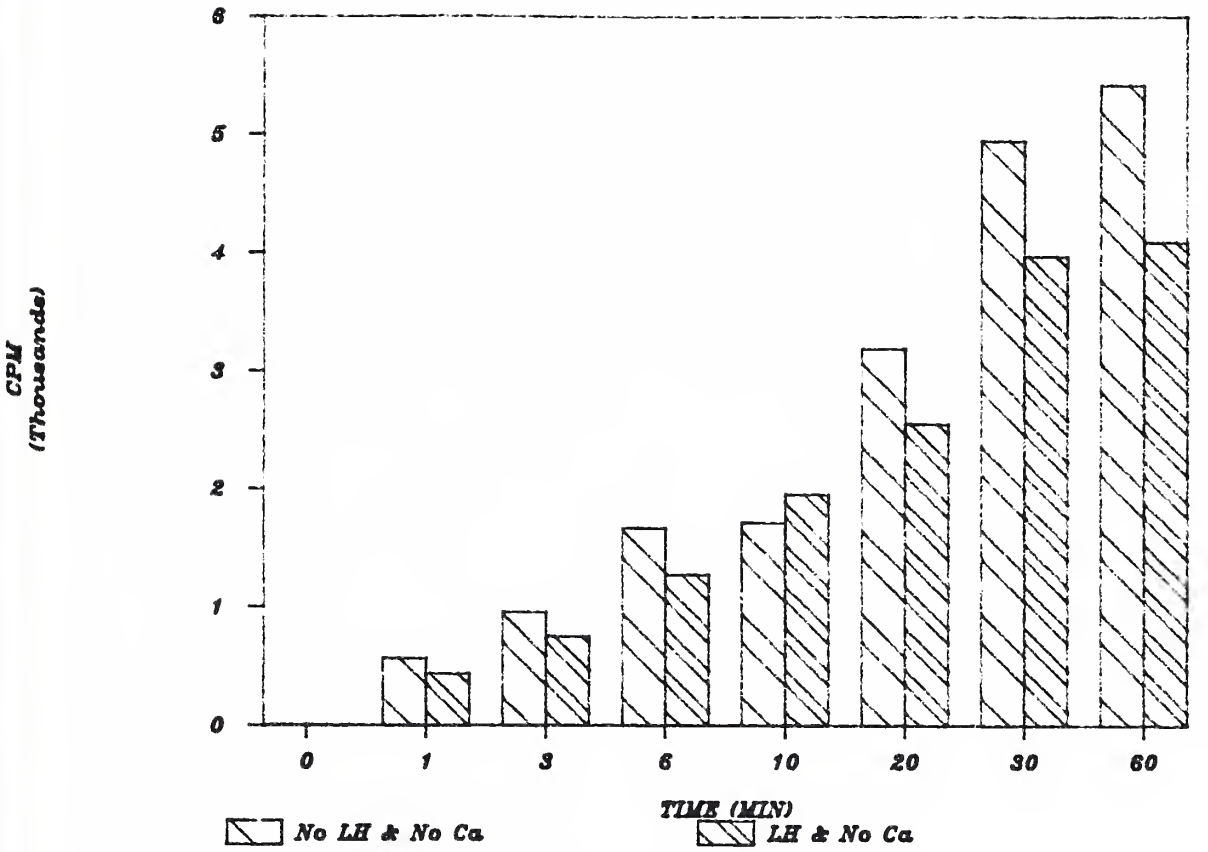

Fig 10b. Binding of ^{32}P-GTP to luteal membranes: in the presence or absence of LH.

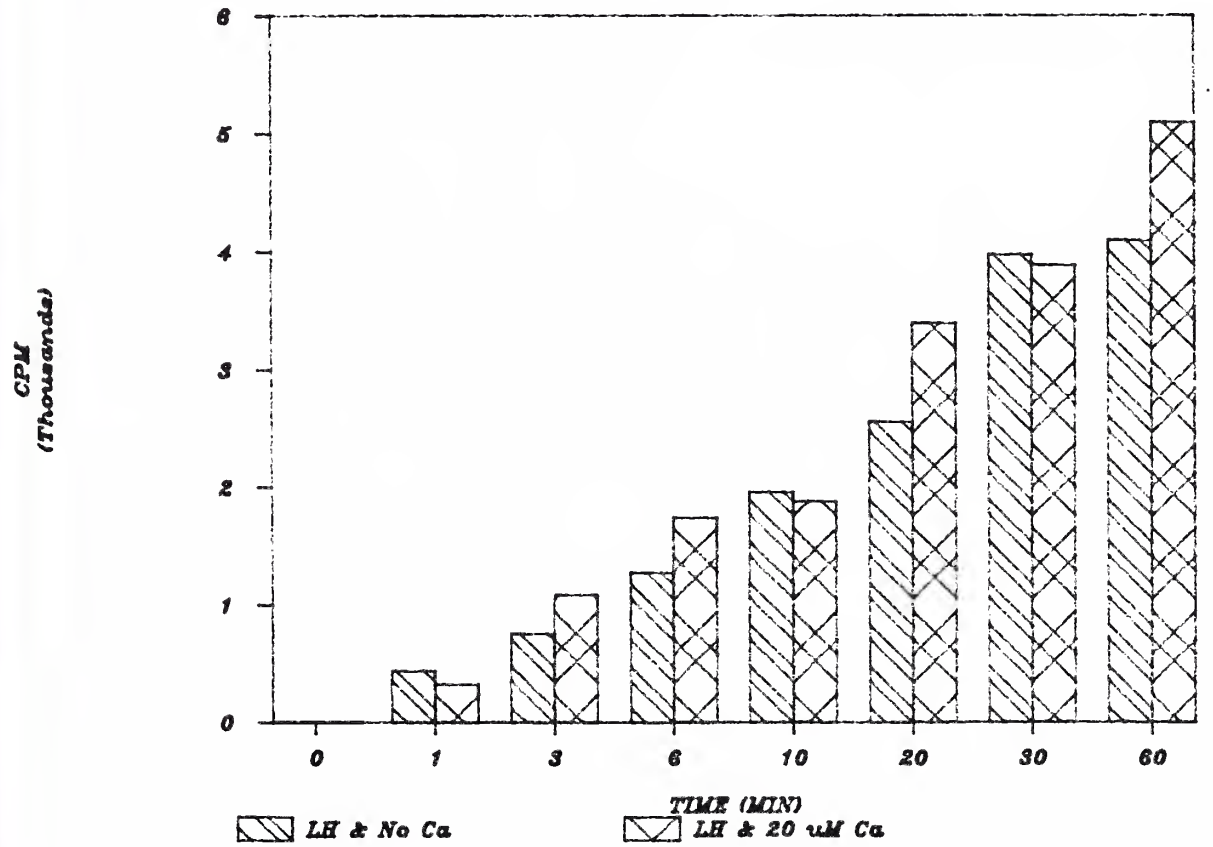

Fig 10c. Binding of ^{32}P-GTP to luteal membranes: in the presence presence or absence of Calcium and with LH.

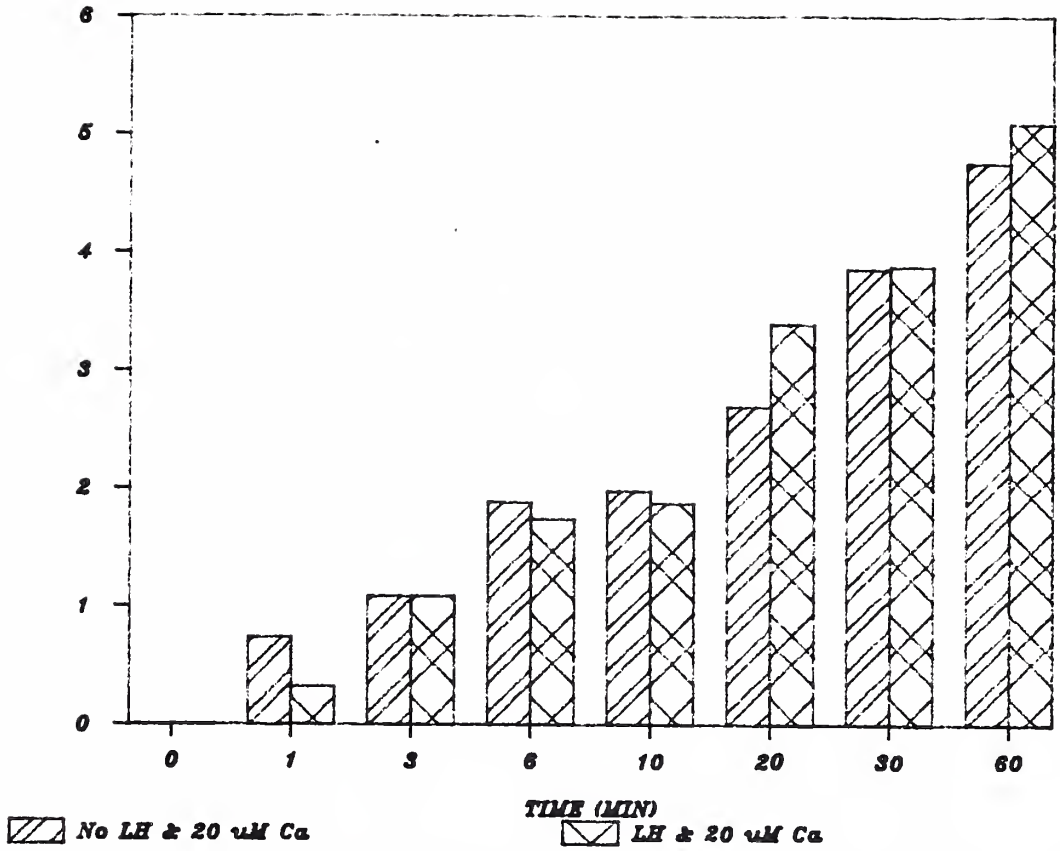

Fig 10d. Binding of ^{32}P-GTP to luteal membranes: in the presence or absence of LH and of Calcium.

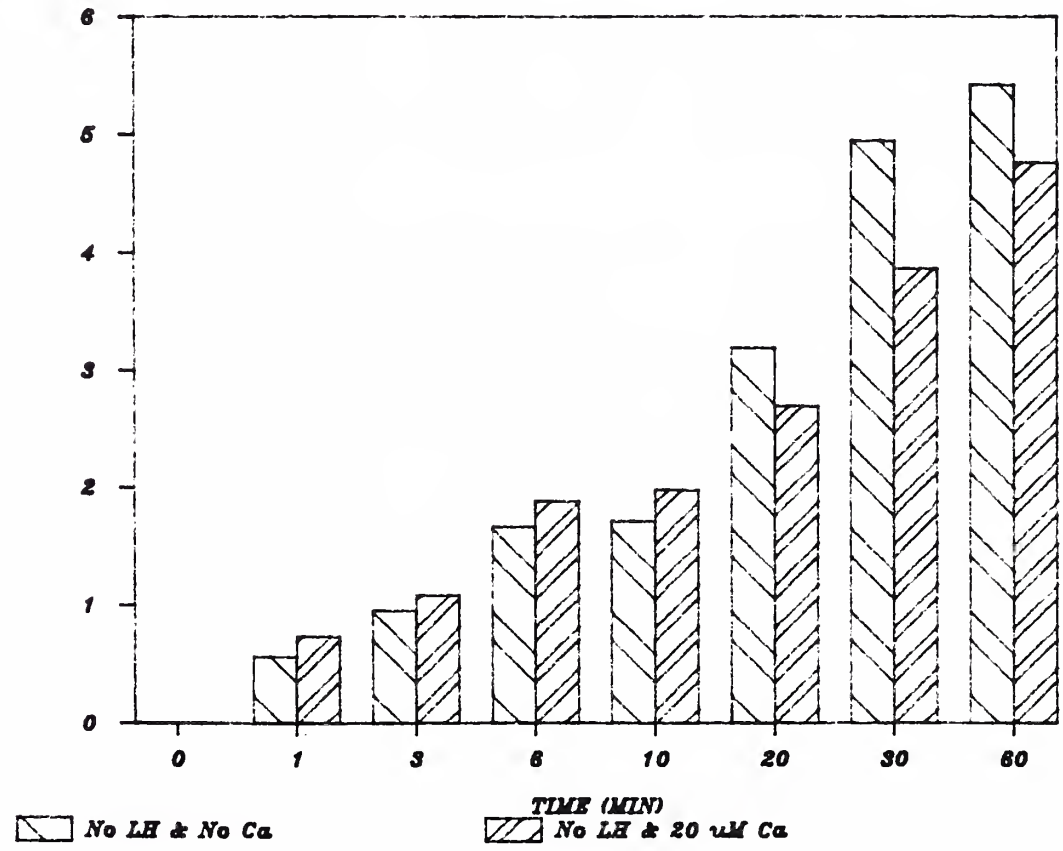

Fig 10e. Binding of ^{32}P-GTP to luteal membranes: in the presence or absence of Calcium and without LH.

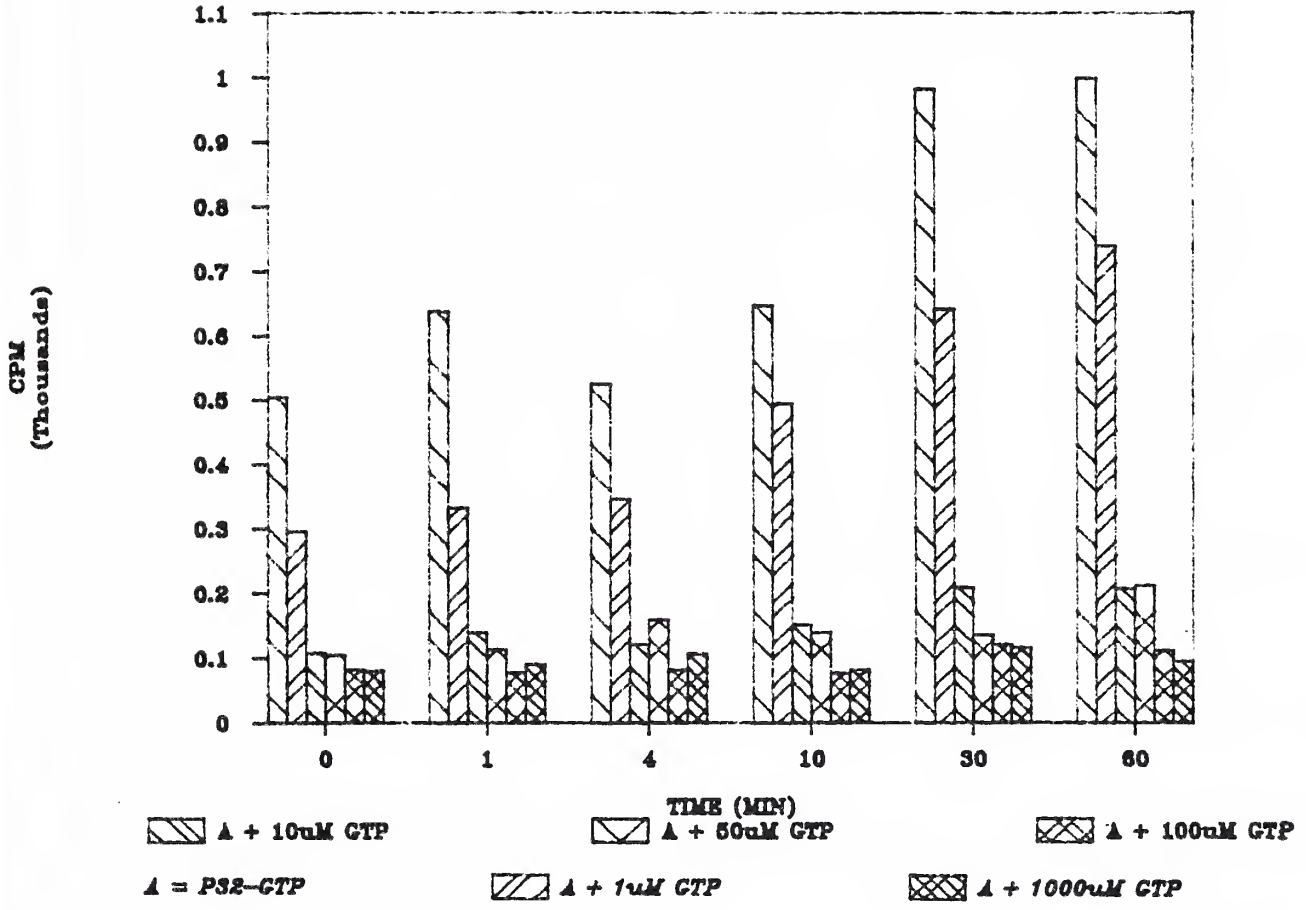

Figure 11. ^{32}P-GTP filter binding assay with increasing concentrations of unlabelled GTP.

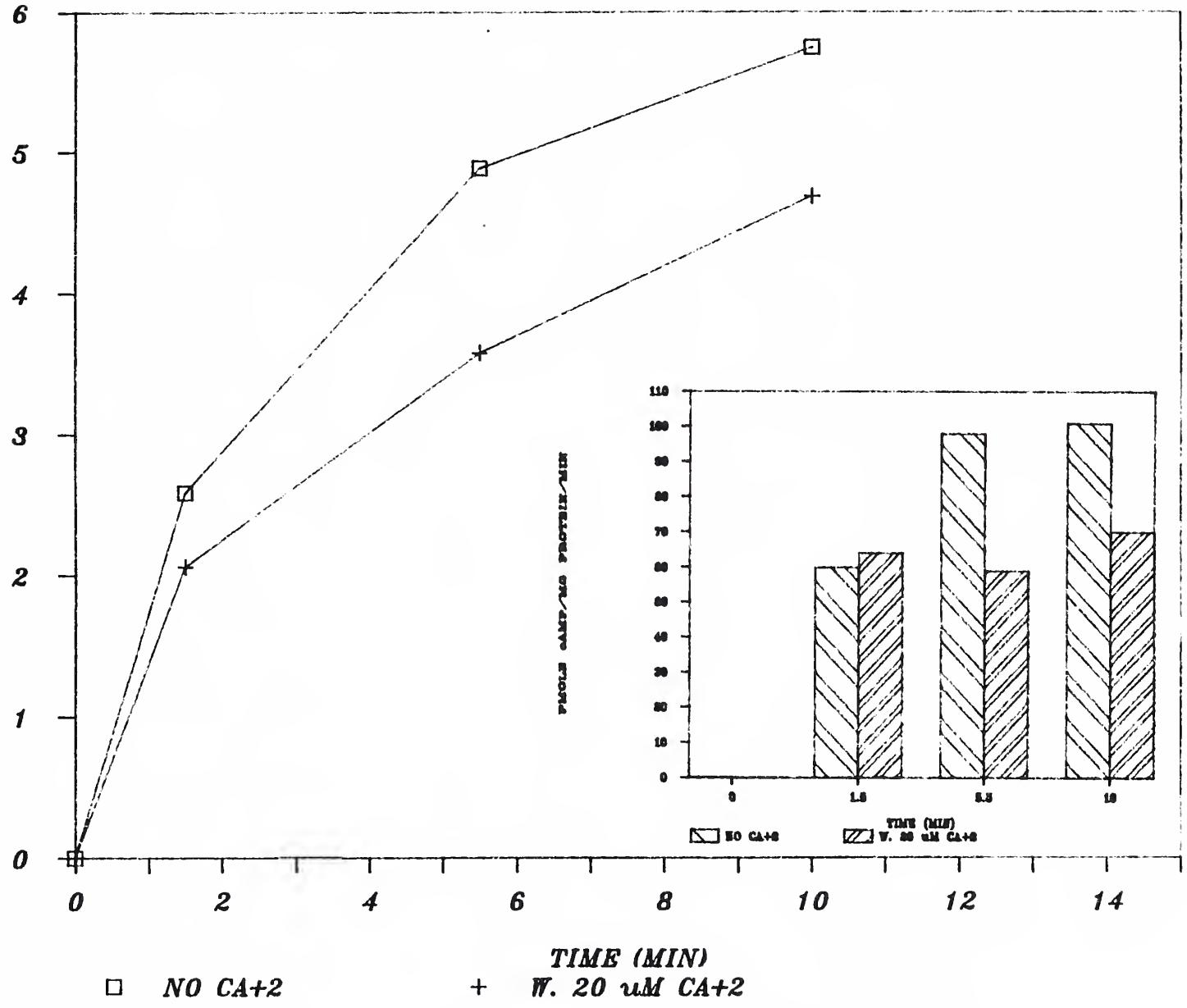

Figure 12. Effect of Ca^{+2} on LH-stimulated ^{3}H-GppNHp release from luteal membranes. Insert: cAMP synthesis during the release.

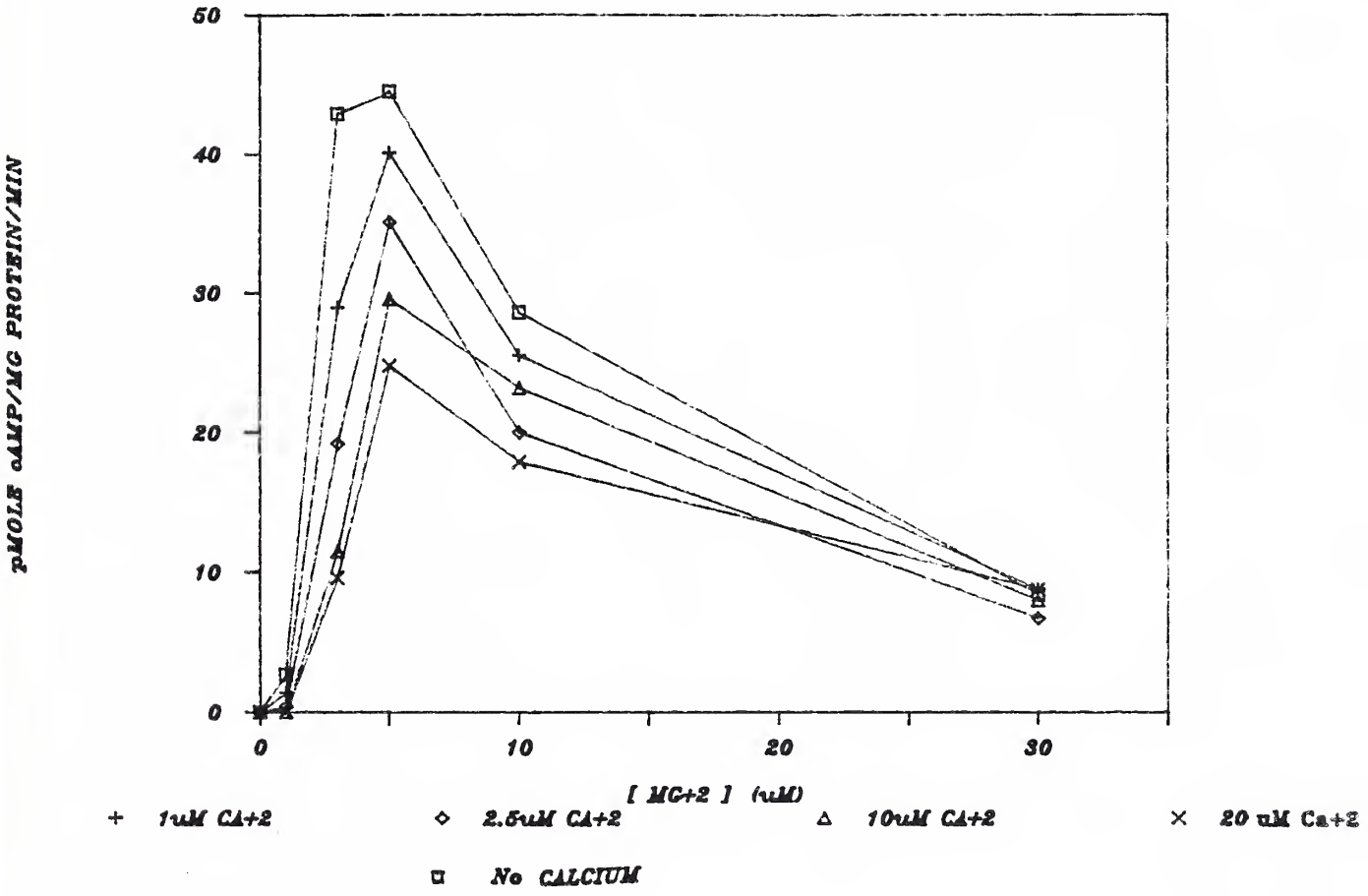

Figure 13. Effect of Mg^{+2} on Ca^{+2}-inhibition of luteal adenylate cyclase activity.

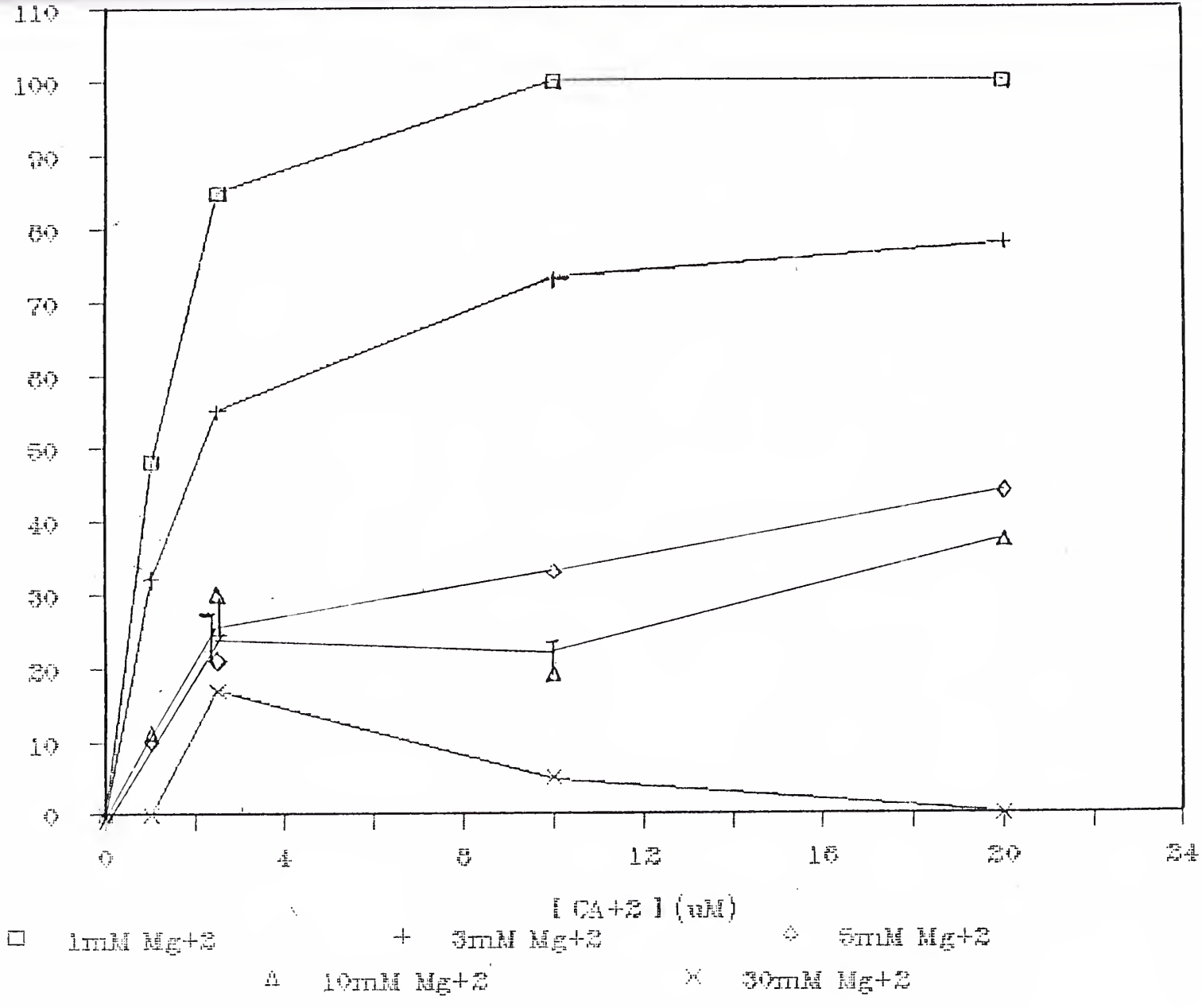

Figure 14. Effect of Mg^{+2} on the degree of inhibition by Ca^{+2} on luteal adenylate cyclase activity.

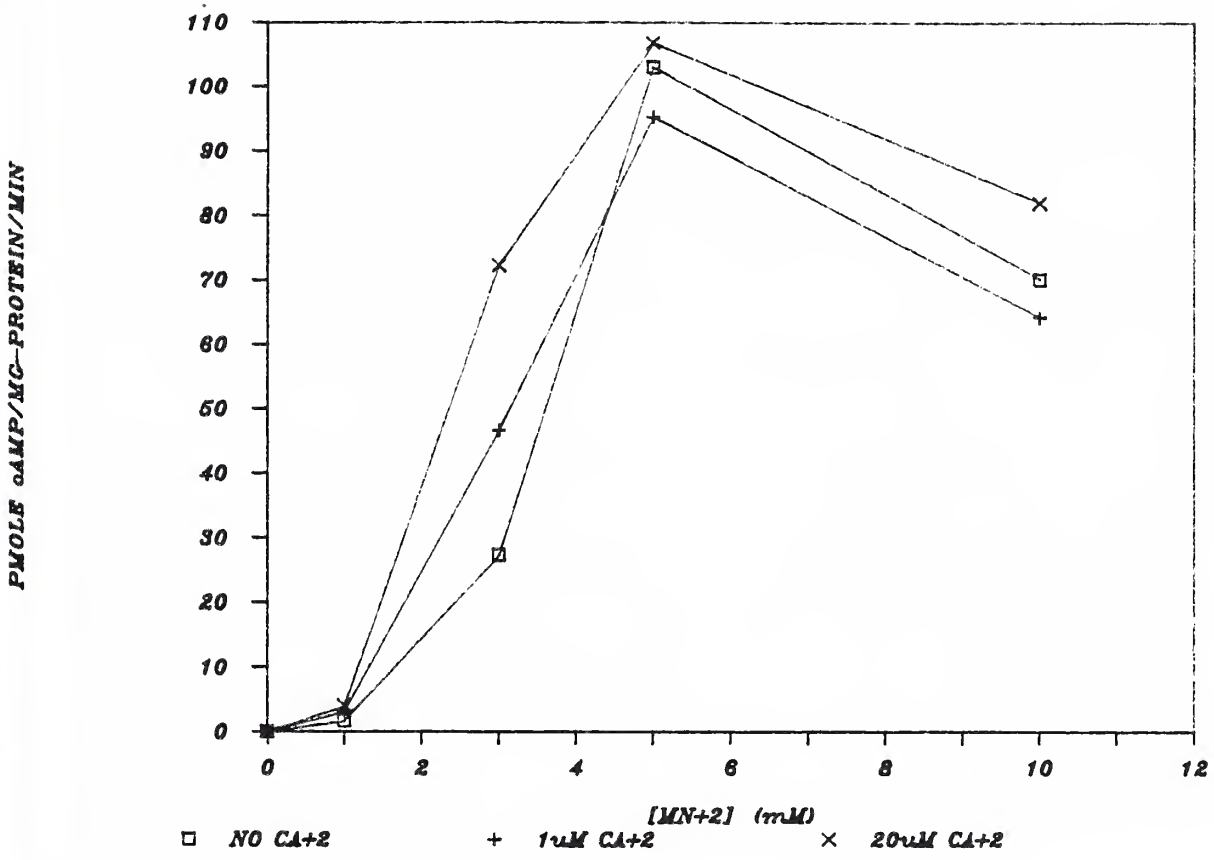

Figure 15. Effect of Mn^{+2} on Ca^{+2}-inhibition of luteal adenylate cyclase activity in the absence of Mg^{+2}.

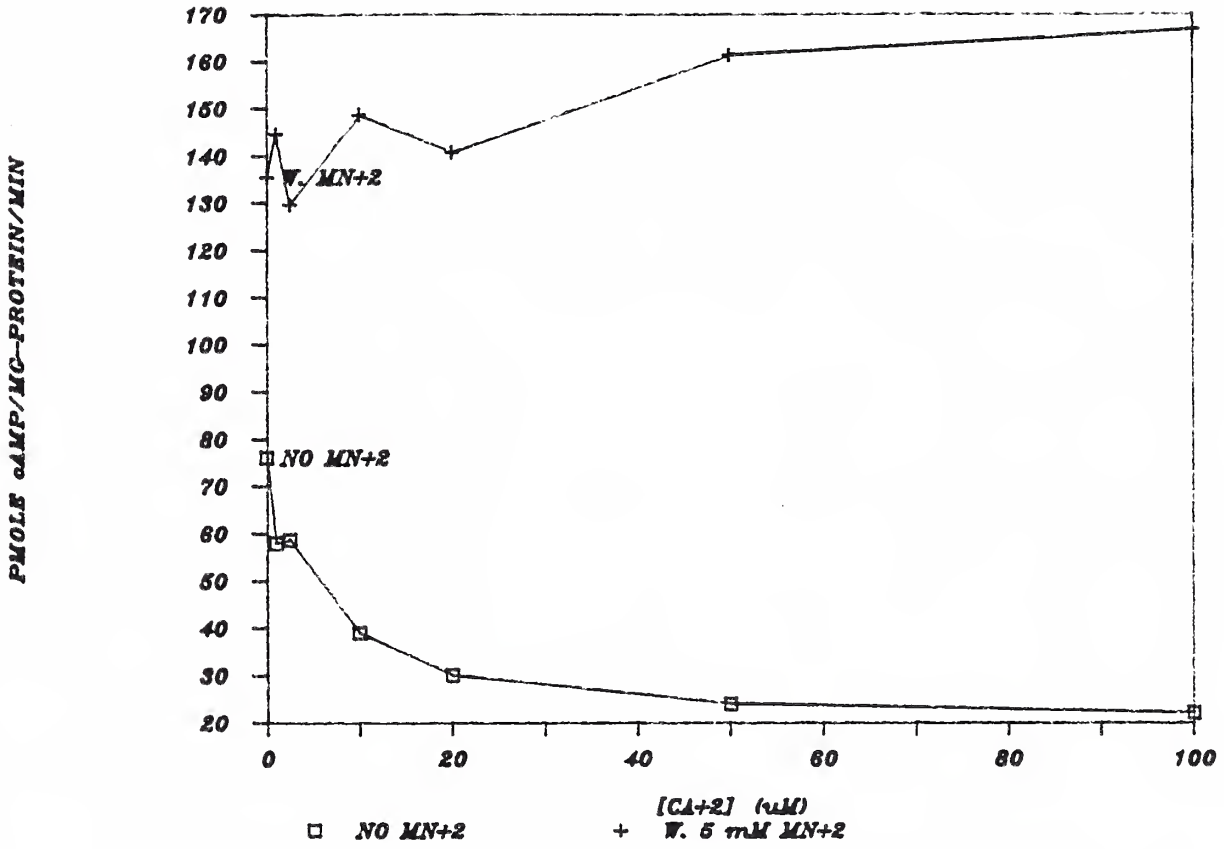

Figure 16. Effect of Mn^{+2} on Ca^{+2}-inhibition of LH-stimulated adenylate cyclase activity.

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