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Evidence for the Regulation of Luteinizing Hormone-Sensitive Adenylate Cyclase By Mono- and Divalent Cations

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements For the Degree of Doctor of Medicine

Steven David Gore

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ABSTRACT

Evidence for the Regulation of
Luteinizing Hormone-Sensitive
Adenylate Cyclase
By Mono- and Divalent Cations

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The actions of pharmacologic agents which alter cellular cation levels were examined in dispersed, enriched rat luteal cells to determine to what extent changes in transmembrane ion gradients or ion fluxes affected the response of the luteal cell to luteinizing hormone (LH). Ouabain, which depletes sodium and potassium gradients between cells and media by inhibition of the Na^+, K^+-ATPase, monensin, a monovalent ionophore which shows high specificity for sodium, and valinomycin, a potassium ionophore, were found to produce significant, dose-dependent inhibition of LH-stimulated cAMP and progesterone production. Little effect of these agents was seen on unstimulated cAMP and progesterone production. Half-maximal inhibition (ID 50) for ouabain was about 50 uM and maximal inhibition was about 50% (200 μM). For monensin, the ID 50 was about 0.1 uM and maximal inhibition was greater than 60% (1 μM); for valinomycin, the ID 50 was less than 10 nM and maximum inhibition was about 60% (10 nM). Inhibition by ouabain and monensin was not overcome by high doses of LH (1 μg/ml). None of the drugs affected cell viability as judged by trypan blue exclusion or release of lactate dehydrogenase into the medium.

Binding and uptake of human chorionic gonadotropin (hCG) by luteal cells was not affected by drug treatment. Ouabain and monensin showed no inhibition of basal or LH-stimulated adenylate cyclase activity in membrane preparations,

and inhibition of phosphodiesterase by isobutyl methylxanthine did not reverse the inhibitory effects of the three drugs. Ouabain and monensin inhibited progesterone accumulation in response to dibutyryl cAMP, while valinomycin had no effect on post- cAMP events in steroidogenesis. Incubation of cells in medium in which sodium was replaced by choline completely reverse the inhibition due to ouabain or monensin, while valinomycin was still inhibitory in the absence of extracellular sodium, consistent with the known pharmacological actions of these drugs.

Stimulation of luteal cAMP accumulation by cholera toxin was unaffected by ouabain treatment; however, monensin significantly inhibited cholera toxin-stimulated cAMP accumulation. Forskolin, which stimulates mammalian adenylate cyclase systems by interaction with the catalytic subunit, significantly increased luteal cAMP production but had a much less pronounced effect on steroidogenesis. Ouabain and prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$) did not inhibit cAMP accumulation in response to forskolin, although monensin showed significant inhibition. Like PG $F_{2\alpha}$, ouabain appears to inhibit coupling of the hormone-receptor complex to the catalytic subunit of adenylate cyclase. Monensin inhibition appears to be at the catalytic subunit, although proximal sites of inhibition are also possible.

Luteal cell response to LH did not appear to require acute sodium influx as substantial reduction of extracellular sodium (to 32 meq/l) had no effect on LH stimulation; tetrodotoxin (1 μM) was also without effect. Reversal of the sodium gradient by substitution of choline for extracellular sodium led to 30% inhibition of LH-stimulated cAMP accumulation. Depolarization of luteal

cells with high concentrations of extracellular potassium (66 meq/l) did not affect LH-stimulation. Incubation of cells in Ca^{++} -free medium decreased unstimulated cAMP levels and blunted the cAMP response of cells to LH. Neither ouabain nor monensin were inhibitory in the absence of extracellular calcium. Inhibition by low doses of ouabain or monensin was super-additive with low doses of PG $F_{2\alpha}$. It is possible that PG $F_{2\alpha}$ shares a common pharmacological mechanism with ouabain and monensin; this may be an increase in free cytoplasmic calcium. Combinations of maximal doses of PG $F_{2\alpha}$ and maximal doses of ouabain or monensin were less inhibitory than either agent alone. The antigonadotropic effect of PG $F_{2\alpha}$ did not require extracellular sodium.

It is concluded that the response of luteal cells to LH is critically dependent on maintainance of normal ionic gradients. Pharmacologic maneuvers which increase intracellular calcium appear to inhibit the response of luteal adenylate cyclase to LH. Since the LH response is blunted in the absence of calcium, the details of calcium-cAMP interaction in the luteal cell are likely to be complex; such interactions may play a crucial role in luteolysis which is initiated by suppression of LH-stimulated cAMP accumulation and progesterone secretion.

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INTRODUCTION

Maintainance of a secretory endometrium which can support a developing embryo requires the steroid hormone, progesterone, which is synthesized by the cyclically formed corpus luteum. In the absence of fertilization and implantation, serum progesterone concentrations fall, the endometrium is sloughed, and a new estrous cycle begins. The corpus luteum has a critical role in maintaining pregnancy and in regulating the estrous and menstrual cycles. A primary function of this organ is the production of steroid hormones, predominantly progesterone.

The cessation of progesterone secretion and gradual involution of the gland in the absence of pregnancy are essential for the beginning of a new cycle and ovulation. Consequently, the control of the steroidogenic apparatus of the corpus luteum is central to the regulation of mammalian reproductive cycles.

The factors which maintain the corpus luteum vary among species, however, prolactin and luteinizing hormone (LH) are required to a greater or lesser extent in most species (1,2). The rat corpus luteum requires an intact pituitary to maintain steroidogenesis; both hypophysectomy and treatment with 2-Br-ergocryptine, a prolactin secretion inhibitor, prevent progesterone secretion (1,2). Treatment with exogenous prolactin will maintain corpus luteum function after hypophysectomy, however prolactin does not itself stimulate steroidogenesis (1). Prolactin is thought to be a permissive luteotropin, facilitating progesterone production without determining the rate of release of the hormone (2). The cellular mechanism of prolactin action is not well understood; however, prolactin helps maintain levels of cholesterol

ester synthetase and cholesterol esterase, enzymes involved in the maintainance of adequate substrate levels for steroidogenesis (3). Prolactin also inhibits synthesis of 3β -and 20α- steroid dehydrogenases, enzymes which metabolize progesterone to inactive products (4). Prolactin also plays a role in maintaining luteal receptors for LH, enabling the organ to respond to this trophic hormone (5,6).

On days 6-9 of pregnancy, prolactin will no longer sustain corpus luteum function in hypophysectomized rats unless supplemented by LH (1). After 8 days, LH is the predominant luteotropin (1,7). In contrast to prolactin, LH is a stimulatory luteotropin which determines the rate and extent of progesterone production in a direct, dose-dependent fashion (2). Like those of many peptide hormones, the actions of LH are thought to be mediated by cAMP (8). Fulfilling Sutherland's criteria for a cyclic AMP - mediated system (9), LH increases cAMP accumulation by the corpus luteum, and both exogenous cAMP and phosphodiesterase inhibitors stimulate luteal steroidogenesis (8). Membranes prepared from corpora lutea exhibit adenylate cyclase activity which can be stimulated by LH (10).

Although cyclic AMP mimics the steroidogenic effects of LH, the precise relationship between LH, cAMP, and luteal steroidogenesis remains unclear. Marsh and colleagues demonstrated a dissociation between the dose-response curves for the stimulation of progesterone accumulation and cAMP accumulation by LH (11). In particular, progesterone accumulation was stimulated by a concentration of LH which was a full order of magnitude less than that required to detect a significant increase in cAMP accumulation. Similar dissociation of cAMP and steroid accumulation has been noted for ACTH

stimulation of the adrenal, LH stimulation of testis, and TSH stimulation of thyroid (8). Three mechanisms have been invoked to explain this dissociation. The spare receptor hypothesis postulates that only a small percentage of hormone receptors need to be occupied for a fractional activation of adenylate cyclase to maximally stimulate progesterone accumulation. The bulk of receptors and adenylate cyclase molecules are, in effect, a spare pool, guaranteeing that the cell will respond to low levels of hormone. The second hypothesis is that hormone binding induces formation of a small amount of cAMP which is not detectable above baseline levels, but which is confined to a small intracellular compartment with priveledged access to steroidogenic machinery. The third hypothesis states that cAMP-dependent protein kinase can be activated by an undetectable increase in cellular cAMP (8,11,12). This issue has not been resolved experimentally, and it remains possible that there are other intracellular messengers which mediate the steroidogenic effects of LH. It is also conceivable that cAMP formed at higher doses of hormone serves other, as yet unidentified, cellular functions.

The production of cyclic AMP is the end result of interaction of a complex of integral membrane proteins, together known as adenylate cyclase, and currently thought to consist of three components. The catalytic moiety, responsible for enzymatic conversion of ATP to cAMP, is inactive unless activity is conferred by a second protein, know as G/F or N. This "coupling protein" possesses GTPase activity and is the site of activation of adenylate cyclase by fluoride ion and by cholera toxin. In the presence of guanine nucleotides, N enables the hormone-receptor complex to activate the catalytic

subunit. There is considerable debate about the details of the coupling mechanism (13).

Although early studies suggested that the luteal cyclase system might differ from other mammalian cyclases in significant ways, the luteal enzyme now appears to be quite similar. Like other cyclases, luteal adenylate cyclase in membrane preparations demonstrates a requirement for ATP and magnesium ion (10). The luteal enzyme exhibits considerable basal activity and is stimulated 2-4 fold by LH, prostaglandin E_1 and epinephrine (10). In contrast to other cyclase systems, a requirement for guanine nucleotides for hormonal stimulation was not demonstrated originally (10). More recent studies have demonstrated that guanine nucleotides control basal activity of luteal adenylate cyclase as well as the responsiveness of the enzyme to hormones. In the absence of guanine nucleotides, LH, human chorionic gonadotropin (hCG), and isoproterenol caused only a marginal stimulation of cyclase, whereas addition of GTP or GTP analogues led to a 3-4 fold stimulation by hormones (14). Additionally, early studies concluded that hormonal sensitivity of the luteal cyclase showed marked dependence on ATP concentration (10); recent studies indicate that this effect is due to the buffering of the magnesium concentration by ATP. Activation of luteal cyclase appears to be extremely sensitive to the concentration of free magnesium ion; addition of substances such as ATP and inorganic phosphate which act as magnesium buffers in the 10- 100 uM range permit stimulation of cyclase over a broader range of added $MgCl_2$ (14). The effects of LH and isoproterenol on cyclase activity are not additive. This indicates that there is only one form of catalytic subunit of luteal cyclase, which is activated by various occupied hormone receptors (14).

Although it is presumed that luteal steroidogenesis is stimulated by a cAMP-dependent protein phosphorylation, the details of this process are not completely understood. Attention has focussed on two loci for stimulation of steroid production: the availability of free cholesterol to act as a substrate for mitochondrial pregnenolone production, and the rate of the cholesterol side chain cleavage reaction (CSCC) which forms pregnenolone from cholesterol. CSCC is the rate-limiting reaction in steroidogenesis (15). Behrman and Armstrong demonstrated an increase in cholesterol esterase activity in luteinized ovaries from rats treated with LH (16). Administration of LH reduced luteal cholesterol ester concentration in control rats and in rats treated with aminoglutethimide, an inhibitor of CSCC (17). This indicated that LH could mobilize substrate stored as cholesterol ester, even when CSCC was inhibited, preventing metabolism of cholesterol. Caffrey and colleagues showed that luteal cholesterol esterase activity in cell homogenates was stimulated by dibutyryl cAMP ($(Bu)_2$cAMP) in the presence of magnesium and ATP, suggesting that the esterase was stimulated by an endogenous protein kinase (18). In addition, cholesterol esterase activity measured during the estrous cycle correlated well with serum progesterone levels and luteal tissue progesterone (18).

Curon et al solubilized the cytochrome P450 system from bovine corpora lutea mitochondria and reconstituted the CSCC using purified components from adrenal cortex. Addition of protein kinase partially purified from bovine corpora lutea increased conversion of cholesterol to pregnenolone in the presence of ATP and cAMP (14). Downing and Dimino added partially purified protein kinase from a crude preparation of CSCC complex from porcine luteal

mitochondria and noted an increase in cholesterol conversion activity (20). Mori and Marsh studied cholesterol metabolism in mitochondria of rat corpora lutea. In rats treated with aminoglutethimide, LH treatment increased mitochondrial cholesterol, in agreement with previous studies. In the absence of inhibitor, LH decreased cholesterol levels, suggesting that the hormone also promoted cholesterol metabolism. An increase in CSCC activity after LH treatment was indeed observed. These investigators further demonstrated that the initial rate of cholesterol conversion did not correlate with mitochondrial cholesterol concentration. In addition, the rate of conversion was slowed markedly without depletion of the majority of mitochondrial cholesterol. The authors postulate that LH treatment not only increased mitochondrial cholesterol and CSCC activity, but increased the fraction of cholesterol which was available for steroidogenesis (21).

Regression of the corpus lutuem, or luteolysis, is marked by a sharp decrease in serum progesterone, and increased secretion of its metabolite 20α-hydroxyprogesterone. Biochemical signs of luteolysis are followd by histological changes indicative of organ involution (22). Luteolysis in the rat can be induced by administration of prostaglandin $F_{2\alpha}$ (PG $F_{2\alpha}$), and there is considerable evidence that PG $F_{2\alpha}$ is the predominant luteolytic signal in the rat and many other species (23). PG $F_{2\alpha}$ is thought to be synthesized in the uterus and transferred to the ovary via counter-current exchange between the uterine vein and ovarian artery (23). There is evidence, however, that the rat corpus luteum contains prostaglandins and can synthesize them (24,25). It is possible that both organs contribute physiologically important prostaglandins.

PG $F_{2\alpha}$ antagonizes the ability of luteal cells to increase cAMP accumulation in response to luteinizing hormone. This was initially shown by Grinwich et al who demonstrated that coincubation of rat luteal slices with PG $F_{2\alpha}$ and LH diminished the rise in cAMP seen with LH alone (26). These results were confirmed by Lahav et al. working with intact corpora lutea in culture (27). In isolated luteal cells, PG $F_{2\alpha}$ stimulates basal progesterone accumulation but antagonizes the ability of LH to increase cAMP and progesterone accumulation (28). PG $F_{2\alpha}$ also diminishes progesterone accumulation in response to $(Bu)_2^{-}$cAMP, indicating that PG $F_{2\alpha}$ inhibits steroidogenesis at two loci: formation of cAMP, and post-cAMP steroidogenesis (29). PG $F_{2\alpha}$ completely inhibits steroidogenesis in response to cholera toxin but only partially reduces cAMP accumulation in response to the toxin (30).

PG $F_{2\alpha}$ does not affect binding of [^{125}I]-hCG to luteal cells (29) nor does it affect the ability of LH to stimulate adenylate cyclase in luteal membrane preparations (31). When membranes were prepared from corpora lutea which had been incubated in vitro with PG $F_{2\alpha}$ for 15 to 60 minutes, both basal and LH-stimulated adenylate cyclase activity was reduced; moreover, fold-stimulation by LH was reduced by approximately one half (32). Thus, while PG $F_{2\alpha}$ does not appear to have a direct effect on LH-stimulated cyclase in membrane preparations, the prostaglandin may induce cellular changes which modulate the responsiveness of the enzyme complex to LH. In addition to the biochemical changes seen in response to acute treatment with PG $F_{2\alpha}$, later changes induced by administration of prostaglandin to rats include decrease in uptake of hCG and prolactin by corpora lutea (6), a

decrease in LH receptors (33), and decreased blood flow to the corpus luteum (34).

Other factors are known to modulate the responsiveness of the corpus luteum to LH. Clayton et al reported that an agonistic analog of gonadotropin releasing hormone (GnRH) was bound specifically to dispersed rat luteal cells and inhibited hCG-stimulated progesterone secretion (35). Subsequent studies by Behrman et al. demonstrated that GnRH and agonistic analogs antagonized LH-stimulated cAMP and progesterone accumulation in rat luteal cells (36). Like prostaglandin, GnRH did not affect hCG binding to luteal membranes or LH-stimulated adenylate cyclase in membrane preparations (36). LH-stimulation of cAMP and progesterone accumulation has been found to be amplified by adenosine and other purines (37). Adenosine had little effect in the absence of LH and was able to reverse PG $F_{2\alpha}$ inhibition in an apparently competitive manner (38). Unlike purine stimulation of adenylate cyclase in other cell types in which adenosine stimulates cyclase via a membrane receptor exposed to the extracellular space (39), adenosine stimulation in luteal cells requires intracellular uptake of adenosine (37). The dose-response and time course characteristics of purine amplification of LH action correspond to the ability of these compounds to stimulate cellular ATP levels, suggesting that increasing substrate availability for cyclic AMP synthesis may be one mechanism by which purines exert their effect (40). The physiologic importance of GnRH and purine modulation of luteal function is still speculative.

The response of the luteal cell adenylate cyclase and steroidogenic apparatus to luteinizing hormone is clearly modulated by many factors. The

plasticity of the luteal cell response to LH may well confer upon the corpus luteum its ability to regulate the post-ovulatory events of the mammalian reproductive cycle. The serum levels of LH and prolactin do not decrease at the time of physiologic luteolysis (41); local factors must be responsible for the early stages of luteolysis.

The mechanisms by which the paracrine regulators modulate the cellular response to LH are unknown. As discussed above, neither PG $F_{2\alpha}$ nor GnRH affect hCG binding to the LH receptor, nor do they affect stimulation of adenylate cyclase by LH in membrane preparations. Further, incubation of luteal tissue with PG $F_{2\alpha}$ before preparation of membranes decreases LH-stimulated cyclase activity in membranes. It would appear that cellular integrity is required for modulation of LH stimulation. X-ray diffraction studies of rat and bovine microsomal membranes prepared from corpora lutea have shown that in vivo treatment with PG $F_{2\alpha}$ leads to an increase in the transition temperature of the membranes, indicative of a decrease in membrane fluidity (42,43,44). The authors of these papers have postulated that PG $F_{2\alpha}$ decreases membrane fluidity, thereby restricting lateral diffusion of membrane proteins and perhaps preventing interaction of the three components of adenylate cyclase. How this apparent fluidity change arises is uncertain; lipid composition of the membranes was apparently unchanged (43). Confirmation of membrane fluidity changes upon PG $F_{2\alpha}$ treatment in cells would help clarify whether this might be an important mechanism in PG $F_{2\alpha}$-induced luteolysis.

Dorflinger and Behrman have reported that treatment of luteal cells with the calcium inophore A23187 inhibits cAMP and progesterone accumulation in

response to LH. Additionally, millimolar doses of calcium inhibit LH-stimulated cAMP accumulation in membrane preparations (31). These investigators have suggested that PG $F_{2\alpha}$ action is mediated by calcium influx or by mobilization of intracellular calcium. Substantial evidence indicates that pituitary gonadotropin release induced by GnRH is mediated by calcium influx (45). Although GnRH mimics the inhibitory actions of PG $F_{2\alpha}$ on luteal cells, experiments with slices of luteal tissue have suggested that depolarization of luteal cells actually increases steroidogenesis in a calcium-dependent manner (46). In porcine granulosa cells, removal of extracellular calcium diminishes LH-stimulated cAMP and progesterone secretion, and treatment with A23187 augments LH-stimulated steroidogenesis (47).

The experiments in this thesis were designed to further elucidate the importance of transmembrane ion flux and membrane potential to the response of luteal cells to LH. The effects of three pharmacologic agents which perturb normal ionic gradients have been studied. The cardiac glycoside ouabain, by inhibiting the Na^+, K^+ -ATPase, allows sodium and potassium to diffuse according to their electrochemical gradients. Monensin is a monovalent cationophore with high selectivity for sodium (48). Valinomycin is a potassium ionophore (48).

MATERIALS AND METHODS

ANIMALS

Immature (26-27 days old) female rats (CD strain, Charles River Laboratories, Wilmington, Massachusetts) were injected subcutaneously with 50 IU pregnant mare serum gonadotropin (Gestyl, Organon Pharmaceuticals, West Orange, New Jersey). Sixty hours later, 25 IU human chorionic gonadotropin (hCG) (A.P.L., Ayerst Laboratories, Rouses Point, New York) was injected.

DISPERSION, ENRICHMENT AND INCUBATION OF LUTEAL CELLS

Isolated luteal cells were prepared as described by Thomas et al. with some modifications (28). All media used for cell dispersion and incubation contained bovine serum albumin (Fraction V, Calbiochem, La Jolla, California) at a concentration of 0.1%. Ovaries were removed 4-6 days following hCG injection, minced by razor and dispersed in 5 ml calcium-free MEM (Medium 1, 1380, Grand Island Biological Company, Grand Island, New York), containing 2000 IU collagenase (Worthington Biochemicals Corporation, Freehold, New Jersey) and 3000 IU deoxyribonuclease (Worthington) per g tissue for 1 h at 37^{o} under 95% O_2 - 5% CO_2. The cells were centrifuged for 5 min. at 100 X g and resuspended in medium 1 containing EDTA (1.1 mM) for two minutes and recentrifuged. The supernatant was discarded, the cells were resuspended in medium 1 and filtered through nylon mesh (Nyten, Tetko, Inc., Elmsford, New York). The cells were again centrifuged for 5 min. and the pellet resuspended in 1.5 ml medium 1.

The luteal cell population was enriched by centrifugation on a discontinuous density gradient (Percoll, Pharmacia Fine Chemicals, Uppsala,

Sweden) at room temperature as described (36). The cells present within the density layers of 1.018 and 1.003 g/ml were aspirated and pooled and washed with 12 ml medium 1, then centrifuged at 100 X g for 10 min. The cells were resuspended in MEM with 25 mM HEPES and Earles' salts (medium 2, 2360, Grand Island Biologicals Co.). Based upon size and lipid inclusions, luteal cells comprised more than 80% of total cells and virtually no blood cell contamination was evident. Cell number was determined with a hemacytometer and cell viability was greater than 90% as assessed by the trypan blue test (49).

Cells were incubated in 12 X 75 mm glass culture tubes at 8 X 10^4 to 1.2 X 10^5 cells/tube in a final volume of one ml medium 2 at 37^o under an atmosphere of 95% O_2-5% CO_2. Additions of hormones and drugs as indicated in the text were made in medium 2. After various incubation times the culture tubes were immersed in a boiling water bath for 10 min. and stored at -20^oC until analyzed.

In experiments in which the sodium concentration was altered, medium 2 was prepared in which all sodium salts were replaced isotonically with choline chloride. The composition of other salts, amino acids and vitamins was identical to MEM with Earle's salts (medium 2). Cell suspensions were divided into aliquots and centrifuged at 100 X g for 5 min. The medium was discarded, and the cells were resuspended in prepared media with various sodium concentrations. After incubating for 5 min at 37^o, the cells were recentrifuged, and the washing procedure repeated.

ADENYLATE CYCLASE ASSAY

LH-sensitive adenylate cyclase activity was assayed essentially as described by Birnbaumer (20) with modification for RIA as described earlier (37). Membranes were prepared from ovaries removed 7-8 days following hCG injection by homogenization at 0^{o}C in a buffer containing 27% sucrose, 1 mM EDTA, 10 mM Tris HCl, pH 7.5, at 5 ml/g tissue. The homogenate was filtered through nylon mesh (Nyten, Tetko, Inc.) and centrifuged at 1000 X g for 10 min at 4^{o} C. The supernatant was recentrifuged at 10,000 X g for 30 min. The pellet was resuspended and quick frozen in dry ice-isopropanol and stored at -70^{o} C.

For assay, membranes were thawed and centrifuged at 10,000 X g for 30 min at 0^{o} C. The pellet was resuspended at 7 ml/g tissue and 40 µl membrane suspension was incubated in a final reaction mixture volume of 100 µl. The final concentrations of reagents were: 10.8% sucrose, 4 mM Tris HCl, 15 mM bis-tris-propane, pH 7.5, 1 mM EDTA, 20 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, 0.1 mg/ml myokinase, 2 mM $MgCl_2$, 0.01% bovine serum albumin. Additions of other drugs and hormones are indicated in the text. The reaction was initiated by addition of ATP (final concentration 3 mM) and incubated at 37^{o}C for 10 min. The incubation was terminated by placing tubes in boiling water for 10 min. Aliquots were removed and assayed for cAMP by RIA as indicated below.

ASSAYS OF cAMP AND PROGESTERONE

The RIA of cAMP was based on the procedure of Steiner (50) using a kit, (Schwartz/Mann, Orangeburg, New York). The assay was sensitive to 0.1 pmol

with minimal cross reaction to other nucleotides (ATP, 0.0001%, AMP 0.001%, cGMP 0.01%). Progesterone production was assayed by RIA as described previously (51).

BINDING AND UPTAKE OF [^{125}I]-hCG TO LUTEAL CELLS

Binding studies were performed as described earlier (36). [^{125}I]-hCG was prepared by the lactoperoxidase method as described earlier (33). Tracer was diluted in medium 2 and added to 1-3 X 10^5 cells in a final volume of 0.5 ml in 12 X 75 mm glass culture tubes. Other additions are described in the text. Cells were incubated for 3 h at 37°C under 95%O_2-5% CO_2; 2 ml medium 2 was added to each tube, and the cells were centrifuged at 100 X g for 5 min. The supernatant fraction was aspirated and the radioactivity in the pellet determined. Non-specific binding was defined as radioactivity associated with cells incubated in the presence of 1000 mIU hCG.

OTHER ASSAYS

Lactate dehydrogenase activity was assayed in medium or in cells lysed hypotonically by the spectrophotometric assay of Wacker (52) using a kit (Statzyme, Worthington). Cell viability was also assessed using the trypan blue method (49). Protein was assayed by the Bradford method (53).

HORMONES, DRUGS, AND REAGENTS

Ovine LH (NIADDK - oLH 23)was a gift from NIH (Bethesda, Maryland). oLH was dissolved in 10 mM Tris HCl, pH 7.5, with 0.1% BSA, at 100 ug/ml. Aliquots were stored at -70°C. hCG (A.P.L., Ayerst Laboratories) was

dissolved in 40 mM Tris HCl pH 7.4 with 5 mM $MgSO_4$ at 1000 IU/ml and aliquots were stored at -4°C. Ouabain and valinomycin were purchased from Sigma (St. Louis, Missouri) and monensin was purchased from Calbiochem. Cholera toxin was purchased from Sigma, and forskolin from Calbiochem. Adenylate cyclase assay reagents were from Sigma. PG $F_{2\alpha}$ tromethamine salt was a gift from Dr. John Pike (UpJohn Co., Kalamazoo, Michigan).

4 mM stock solutions of ionophores were prepared in ethanol and stored at -4°C. Ionophores were diluted in medium 2 before addition to cells, and final concentration of ethanol never exceeded 0.01%. Solutions of ouabain were made in medium 2 on the day of the experiment. Forskolin was dissolved in ethanol (10 mM) and stored at -4°C.

STATISTICAL ANALYSIS

Luteal cells from several animals were pooled and samples of equal numbers of cells were exposed to various treatments within an experiment. Data are reported as the mean $\pm$ SEM for four replicates of representative experiments. The two-tailed t test for paired data was used to test differences between samples. Pairs were based on the sequence of replicate incubations within each treatment group.

RESULTS

Preincubation of dispersed, enriched luteal cells with ouabain caused significant, dose-dependent inhibition of LH-stimulated cAMP and progesterone accumulation (Figure 1). Maximal inhibition of LH-stimulated cAMP and progesterone accumulation was about 50% at a ouabain concentration of 200 μM. Half-maximal inhibition occurred at 50 μM ouabain. In Figure 2, dose-response curves of ouabain inhibition of cAMP and progesterone accumulation in the presence of increasing doses of LH are plotted. No consistent effect of ouabain was seen on unstimulated cAMP or progesterone accumulation, although occasional, slight inhibition or stimulation was observed. Ouabain inhibition was greatest at low doses of LH (71.5% at LH = 10 ng/ml) but was still significant at high doses of LH (35% at LH = 1000 ng/ml). A plot of the reciprocal of cAMP accumulation against drug concentration with 10 and 100 ng/ml of LH illustrated that ouabain inhibition of LH-stimulated cAMP accumulation was competitive with a K_i of about 20 μM (90). The estimated K_i for ouabain inhibition of LH-stimulated progesterone secretion was 10-15 μM. This value was obtained from a plot of the reciprocal value of maximal progesterone secretion against the concentration of inhibitor, based on the assumption that inhibition was competitive. Although lower, the K_i for inhibition of progesterone secretion compares reasonably well with the K_i for inhibition of cAMP accumulation. In early experiments, cells were incubated with ouabain for 1 h before the addition of LH, however, identical inhibition was seen with 10 min. preincubation (see Table 4), and in later experiments, 20 min. preincubations of cells with ouabain were used routinely.

Incubation of cells with 100 μM ouabain for 2 h did not affect cell viability as judged by the exclusion of trypan blue or by the release of the cytoplasmic enzyme, lactate dehydrogenase (data not shown). The reversibility of ouabain inhibition was tested in the following manner. Cells were incubated with ouabain (100 μM) for 15 min., the media was removed following centrifugation (100 x g, 5 min.), the cells resuspended in fresh medium with or without ouabain, and incubated for an additional 5 min. The washing procedure was then repeated, the cells were resuspended in media with or without ouabain, LH was added 5 min. after resuspension of the cells, and the incubation was continued for 90 min. As shown in Table 1, inhibition of cAMP accumulation was 67 $\pm$ 7% in the treated samples and 37 $\pm$ 4% in the washed samples. In a separate experiment, inhibition of progesterone accumulation was reduced from 76 $\pm$ 3% in the samples which were washed and retreated to 18 $\pm$ 3% in the washed samples. The differences between washed and retreated samples were significant ($p < 0.05$).

There are several loci at which ouabain might inhibit LH-stimulation, including: binding of hormone to receptor, interaction of hormone-receptor complex to the N subunit of adenylate cyclase, interaction of the subunit with the catalytic moiety of the enzyme, or at the catalytic subunit directly. Inhibition could also be mediated by an increase in phosphodiesterase activity. Additionally, progesterone accumulation could be inhibited at sites distal to cAMP accumulation. Several experiments were performed to assess to possible site of inhibition by ouabain.

First, the effects of ouabain on the binding and uptake of hormone by cells was tested. Cells were incubated with ouabain (100 μM) for 20 min.

before addition of [^{125}I]-hCG and various amounts of unlabeled LH. Incubation and determination of bound radioactivity then proceeded as described in Methods. Treatment with ouabain had no significant effect on the binding of labeled ligand or on competition for binding with unlabeled hormone (data not shown). Ouabain treatment therefore, does not appear to affect interaction of hormone with receptor.

Next, the effect of ouabain on adenylate cyclase activity was studied in membrane preparations as described in Methods. Table 2 demonstrates that ouabain (100 μM) had no significant effect on either basal or LH-stimulated adenylate cyclase activity in membrane preparations.

The possibility that ouabain treatment decreased cAMP accumulation by increasing metabolism of the nucleotide was also considered. Cells were preincubated in the presence or absence of the cyclic nucleotide phosphodiesterase inhibitor isobutyl methylxanthine (IBMX, 100 μM) for 10 min. Ouabain (100 μM) was then added, and incubation continued for an additional 20 min. before the addition of LH (100 ng/ml); the cells were incubated with LH for 90 min. If ouabain treatment depended on an increase in phosphodiesterase activity for its inhibitory effects, treatment with IBMX should reverse the inhibition. In cells treated with IBMX, ouabain inhibition of LH-stimulated cAMP accumulation was 45 $\pm$ 2%. Thus, ouabain did not appear to exert its inhibitory effect through increased metabolism of cAMP.

Although ouabain inhibited cAMP and progesterone accumulation in a parallel manner, it is possible that inhibition of progesterone production occured at a site distal to cAMP accumulation. To test this possibility, cells were treated with the membrane permeable cAMP analogue dibutyryl cAMP

($(Bu)_2$-cAMP) after a 20 min. preincubation with ouabain (100 μM); the incubation was allowed to proceed for 90 min. As shown in Figure 3, progesterone accumulation in response to $(Bu)_2$-cAMP) was significantly inhibited in cells pretreated with ouabain; maximum response to $(Bu)_2$-cAMP was 55 $\pm$ 1.5 ng/10^5 cells in the absence of the drug and 29 $\pm$ 0.5 ng/10^5 cells in its presence.

In most tissues, pharmacologic effects of ouabain are attributed to the glycoside's ability to inhibit the Na^+, K^+-ATPase (54). Inhibition of this pump allows sodium and potassium to diffuse across the plasma membrane according to their electrochemical gradients. Because ouabain treatment diminishes both the sodium and potassium gradients, it was of interest to consider whether selective interference with either gradient similarly affected cellular responses. To this end, two ionophores, monensin and valinomycin, were tested for their ability to inhibit LH-stimulated cAMP and progesterone accumulation. Monensin is a polyether monovalent cationophore with high selectivity for Na^+ transport. Monensin transports K^+ 1/10 as well as Na^+ and also interacts with protons (48). Valinomycin is a cyclodepsipeptide which acts specifically as a potassium cationophore. However, because valinomycin is a neutral compound, valinomycin-K^+ complexes are charged, and potassium diffusion across the cell membrane would be expected to be limited by the Nernst potential for potassium (48).

Figures 4 and 5 show that both monensin and valinomycin inhibit LH-stimulated cAMP and progesterone accumulation in significant, dose-dependent manners. In the experiment displayed in Figure 4, cells were treated with monensin for 30 min. before the addition of LH (200 ng/ml). The

maximal inhibition of cAMP and progesterone accumulation by monensin was greater than 60% at a 1 μM concentration of the drug. The experiment with valinomycin was performed similarly except that LH concentration was 50 ng/ml. Maximum inhibition by valinomycin was 60% at 10 nM. The IC 50 for monensin was about 0.1 μM: for valinomycin, the IC 50 was less than 10 nM. Like ouabain, neither drug had a consistent effect on unstimulated cAMP or progesterone (Figure 6 for monensin, data for valinomycin not shown). Figure 6 shows dose-response curves for monensin inhibition in the presence of increasing doses of LH. As with ouabain, inhibition by monensin was not reversed by increased LH concentration. Monensin inhibition of LH-stimulated cAMP accumulation was more evident at lower doses of LH (34.5% inhibition with 0.1 μM monensin seen at 50 ng/ml of LH) than at higher doses of LH (21% inhibition seen at 0.1 μM monensin at 200 ng/ml of LH). Inhibition by monensin was competitive in nature with a K_i of about 0.06 μM. The estimated K_i for monensin-inhibition of LH-stimulated progesterone secretion was about 0.02-0.04 μM. These values were obtained from plots of reciprocal response against drug concentration as explained above for ouabain.

Neither monensin (0.2 μM) nor valinomycin (2 nM) significantly affected cell viability as judged by trypan blue exclusion or LDH release (data not shown). When tested for reversibility of drug effect in a protocol identical to that described above for ouabain, inhibition of cAMP and progesterone accumulation by monensin (0.2 μM) was almost completely reversed (Table 1). Washing the cells reduced monensin inhibition from 68.4 $\pm$ 6.3% to 2 $\pm$ 0.3% for cAMP and from essentially 100% inhibition of stimulated progesterone levels to super-control levels. Inhibition of cAMP accumulation by valinomycin (2 nM)

was surprisingly enhanced by the washing procedure; washed samples were inhibitied 53.8 ± 3.8% whereas retreated samples were inhibited by only 18.1 ± 1.5%. Washing did, however, substantially reduce valinomycin inhibition of progesterone accumulation from 83 ± 26% to 20 ± 0.9%. All differences were significant ($p < 0.05$).

The effect of a 20 min. preincubation with monensin (0.2 µM) or valinomycin (4 nM) on the binding and uptake of [^{125}I]-hCG by luteal cells was also tested. Neither drug significantly altered the binding of labeled hormone or competition for binding of radiolabeled hormone by unlabeled hormone (data not shown). Table 3 shows the result of an experiment in which the ability of monensin to inhibit the activity of adenylate cyclase in luteal membrane preparations was tested. Like ouabain, monensin (1 µM) did not inhibit basal or LH-stimulated levels of cAMP in this assay system. Valinomycin was not tested in membrane preparations.

Neither monensin nor valinomycin appeared to exert their influence on LH-stimulation by increasing the activity of phosphodiesterase. After a 10 min. preincubation with IBMX (100 µM), cells were treated with monensin (0.2 µM) or valinomycin (2 nM) for 20 min. before incubation with LH (100 ng/ml) for 90 min. Both drugs markedly inhibited LH-stimulated cAMP accumulation in the presence of IBMX: inhibition by monensin was 44 ± 3.4% and inhibition by valinomycin was 28 ± 0.8%.

The ability of these drugs to inhibit progesterone accumulation in response to $(Bu)_2$cAMP was also tested. Cells were preincubated with monensin (0.2 µM) or valinomycin (2 nM) for 20 min. before the addition of $(Bu)_2$cAMP. Monensin significantly inhibited progesterone accumulation in

response to $(Bu)_2$cAMP (Figure 7). Progesterone accumulation in response to 1000 μM $(Bu)_2$cAMP was inhibited 79.1 ± 27.5%. Unlike ouabain and monensin, valinomycin had no significant effect on stimulation by the nucleotide.

In an attempt to evaluate the mechanism of inhibition by the three drugs, it was deemed important to ascertain whether inhibition was dependent on an effect on transmembrane ion gradients. Thus, if inhibition by ouabain was dependent on blockade of the Na^+, K^+-ATPase and a subsequent increase in intracellular sodium, the effect of the drug would be expected to depend on the presence of extracellular sodium. A similar argument can be made for monensin. Valinomycin, whose actions are thought to arise from its capacity to transport K^+ only, should not depend on extracellular sodium for its inhibitory effects. To test the sodium dependence of these drugs, media was prepared in which sodium was replaced isotonically with choline chloride. The composition of amino acids and vitamins were identical to MEM. Figure 8 illustrates the influence of replacing sodium with choline on inhibition by the three drugs of LH-stimulated cAMP accumulation. In this figure, the cAMP response is plotted as % of cAMP levels in cells treated with LH alone (100 ng/ml). Inhibition by ouabain (100 μM) and monensin (0.2 μM) were 24 ± 0.4% and 92.7 ± 7.3%, respectively, in sodium replete media. The inhibition by these drugs was completely reversed when sodium was replaced by choline. The absence of sodium had a much smaller effect on the inhibition by valinomycin (2 nM); cAMP accumulation was inhibited 62 ± 3% in the presence of sodium and 42 ± 2% in its absence. This difference was significant ($p < 0.02$).

The preceeding experiments demonstrated that three drugs which interfere with maintainance of normal transmembrane ionic gradients significantly

inhibited the response of the luteal cell to LH. The drugs appeared to be without effect in membranes preparations and did not affect binding and uptake of hormone by cells. Both ouabain and monensin have as predominant pharmacologic mechanisms the ability to increase intracellular sodium concentrations, although the routes by which the drugs increase sodium levels differ. Like PG $F_{2\alpha}$ (28,29), both drugs inhibited the response to LH at the level of cyclic AMP accumulation. The next set of experiments was undertaken to further localize the molecular site at which ouabain and monensin inhibit cAMP accumulation.

Binding of cholera toxin to mammalian cell membranes causes ADP-ribosylation of the N subunit of adenylate cyclase. This irreversibly activates the subunit which in turn activates the catalytic moiety. Cholera toxin thereby bypasses the hormone receptor (13). Inhibition of cholera toxin-stimulated cAMP accumulation must therefore occur at the N or C subunits. The ability of ouabain and monensin to inhibit cholera toxin-stimulated cAMP was tested. Figure 9 shows the effect of ouabain (100 μM) and monensin (0.2 μM) on cAMP accumulation in luteal cells stimulated by cholera toxin. Cells were incubated with cholera toxin for 2 h. Ouabain had no significant effect on cholera toxin-stimulated cAMP production. Monensin, on the other hand, significantly reduced cAMP accumulation at all doses of cholera toxin tested. Maximal cAMP levels were lowered from 2.4 $\pm$ 0.3 pmol/10^5 cells in the absence of drug to 1.1 $\pm$ 0.2 pmol/10^5 cells with 0.2 μM monensin. The ED 50 for cholera toxin-stimulation was not changed by monensin treatment.

Further localization of the inhibition was provided by experiments with forskolin. This diterpine has been found to stimulate adenylate cyclase in all mammalian cells tested, although stimulation of luteal cell cyclase has not been reported (55). Stimulation by forskolin is thought to result from direct activation of the catalytic subunit of adenylate cyclase, bypassing the hormone receptor and N subunit (56). The ability of forskolin to stimulate luteal cell cAMP accumulation is shown in Figure 10. Like other mammalian cells, luteal cells respond to forskolin with increased cAMP accumulation. Maximal stimulation was 5-fold in response to 100 μM forskolin. It is unclear from the dose-response curve whether higher doses of forskolin would further increase cAMP accumulation; doses were limited by the solubility of the drug in ethanol and the need to maintain final ethanol concentration at 1%. The ED 50 for forskolin was about 20 μM. Interestingly, forskolin stimulation of cAMP was associated with only a slight increase in progesterone secretion. As seen in figure 11, 100 μM forskolin increased cAMP levels from 0.24 ± 0.26 to 3.9 ± 0.2 pmol/10^5 cells, but increased progesterone only from 4.0 ± 0.4 to 5.3 ± 0.2 ng/10^5 cells ($p < 0.05$). In contrast, doses of LH which increase cAMP only slightly, maximally stimulate progesterone production (11,36).

Luteal cells were treated with ouabain or monensin for 20 min. before the addition of forskolin. Incubation was allowed to proceed for 1 h. The ability of PG $F_{2\alpha}$ to inhibit forskolin stimulation was also tested. In this case, PG $F_{2\alpha}$ was added directly before forskolin. PG $F_{2\alpha}$ (210 nM) had no significant effect on forskolin stimulation (Figure 12a): ouabain (100 μM) was also without significant inhibitory effect (Figure 12b). However, monensin (0.2 μM) significantly inhibited forskolin-induced cAMP accumulation; maximum

cAMP levels were reduced from 3.7 ± 0.5 to 1.5 ± 0.3 pmol/10^5 cells by monensin but the ED 50 for forskolin did not appear to be affected by drug treatment.

PG $F_{2\alpha}$ exerts its antigonadotropic action whether added before, simultaneous with, or after LH (27,57). We tested whether the order of addition of drug and hormone was an important factor in expression of the inhibitory activities of ouabain and monensin. Cells were treated with ouabain (100 μM) or monensin (0.2 μM) at various times before or after the addition of LH (200 ng/ml in the case of ouabain, 50 ng/ml for monensin). Control tubes received media at identical time points and the cells were incubated with LH for 90 min (Table 4). If ouabain was added 15 min. before LH, 48% inhibition of cAMP accumultion was seen. However, when added 15 min. after LH, ouabain caused only a 12% inhibition. Later addition of the drug was not effective. Monensin, when added 15 min. before LH, caused 45 ± 21.4% inhibition; inhibition was only 18.5 ± 3.2% when added simultaneously with LH, and 19.6 ± 5.3% when added 15 min. after the hormone. Thus, unlike prostaglandin, inhibition of cAMP accumulation by ouabain was only effective when the drug was added before or simultaneous with LH. The drug was ineffective when added after the hormone. Monensin inhibition was diminished, but not eliminated when added after LH.

It is possible that activation of adenylate cyclase by LH depends on an acute ionic influx or depolarization; inhibition by ouabain and monensin could possibly be explained by prior depletion of the sodium gradient by these drugs. If this were the case, pretreatment of cells with LH would render them refractory to the effects of ouabain and monensin. This possibility was

explored in two ways. First, the dependence of LH-stimulation on the presence of extracellular sodium was tested. If stimulation of adenylate cyclase by LH required influx of sodium, removal of extracellular sodium should inhibit cAMP accumulation in response to the hormone. Luteal cells were incubated in media in which sodium was replaced isotonically with choline chloride as described in Methods. LH (100 ng/ml) was added, and the cells were incubated for 90 min. As seen in Figure 13, reduction of sodium from 128 to 32 meq/l had no significant effect on LH-stimulation of cAMP accumulation. When sodium was completely eliminated from the medium, maximum cAMP accumulation was reduced by 30 $\pm$ 1%. The ED 50 for LH was not affected by sodium concentration.

We also examined the sodium channel blocker tetrodotoxin (TTX) to test for a requirement of sodium influx in response to LH. If LH binding opened a sodium channel and induced sodium influx which may be necessary for maximal activation of adenylate cyclase, tetrodotoxin would then be expected to inhibit the response to LH. Cells were incubated with TTX for 30 min. at which time LH (100 ng/ml) was added, and the cells incubated for an additional 90 min. Doses of TTX as high as 1 μM had no significant effect on basal or LH-stimulated cAMP levels (data not shown).

Ouabain depolarizes the cell because the Na^+, K^+-ATPase, an electrogenic pump, is inhibited resulting in depletion of sodium and potassium gradients. Monensin, by virtue of its action as a sodium ionophore would also be expected to have a cell-depolarizing effect. To ascertain whether depolarization per se inhibited LH-stimulation of cAMP accumulation, luteal cells were incubated in medium in which various concentrations of NaCl were replaced isotonically with KCl. Cells were incubated for 90 min. after the

addition of LH. The results are shown in Figure 14. No significant inhibition was seen with KCl concentrations as high as 66 mM.

Thus, it appears that inhibition of LH-activation of luteal cells by ouabain and monensin is dependent on the presence of extracellular sodium, but the inhibitory effects appear not to be mediated by changes in membrane potential. Pharmacologic actions of ouabain in the heart and other tissues such as thyroid have been ascribed to an increase in intracellular calcium levels produced as a consequence of the drug-induced increase in intracellular sodium. The increase in sodium increases sodium-calcium exchange and it may also inhibit calcium extrusion (58). We therefore tested the dependence of inhibition by the two drugs on the presence of extracellular calcium. Cells were incubated in "Ca^{++}-free" medium (medium 1) to which various levels of calcium were added. Ouabain (100 μM) or monensin (0.2 μM), were added to the cells for 20 min. before the addition of LH (100 ng/ml), and incubation continued for 90 min.

As seen in Table 5, depletion of extracellular calcium reduced the amount of cAMP produced by luteal cells in response to LH. In the absence of added calcium, 3.7 $\pm$ 0.4 pmol accumulated, as compared to a maximum of 14.4 $\pm$ 2.6 at a calcium concentration of 1.5 meq/l. The effects of higher calcium concentrations were not tested. Inclusion of 100 μM EGTA in the incubation media yielded values which were not different from 0 Ca^{++} (data not shown). Unstimulated levels of cAMP accumulation also increased with calcium concentration, rising from 0.96 $\pm$ 0.3 pmol at 0 Ca^{++} to 4.0 $\pm$ 2.0 at 1.5 meq/l. In the absence of calcium, neither ouabain nor monensin inhibited cAMP accumulation, however both drugs elicited significant inhibition when calcium

was replaced. At 1.5 meq/l, ouabain inhibition was 90.4 ± 0.7%. Inhibition by monensin was not significant at 0.5 meq/l Ca^{++}, but was below unstimulated levels at 1.5 meq/l. In preliminary experiments, the effect of calcium depletion on valinomycin inhibition was tested. Valinomycin (4 nM) did not inhibit LH-stimulated cAMP accumulation in the absence of calcium, but was maximally inhibitory (80%) at 0.5 meq/l Ca^{++} (data not shown).

Because inhibition by ouabain resembled the antigonadotropic effect of PG $F_{2\alpha}$, it was of interest to determine whether inhibition by ouabain or monensin was additive with prostaglandin. Cells were treated with ouabain (50-100 μM) or monensin (0.1-0.2 μM) for 20 min. at which time various doses of PG $F_{2\alpha}$ were added and LH (100 ng/ml) added immediately thereafter (90 min.). The results are shown in Figure 15. In the absence of other drug treatment, PG $F_{2\alpha}$ inhibited the LH-stimulated accumulation of cAMP with an ID 50 of 21 nM. At 210 nM, cAMP accumulation was reduced to unstimulated levels. Addition of a submaximal concentration of ouabain (50 μM) shifted the dose-response curve for PG $F_{2\alpha}$ to the left, causing maximal inhibition at a concentration of prostaglandin of 2.1 nM. Similarly, the presence of monensin potentiated the inhibition produced by PG $F_{2\alpha}$, with maximal inhibition at 2.1 nM PG $F_{2\alpha}$ (0.1 μM monensin). PG $F_{2\alpha}$ also changed the dose-response characteristics of ouabain and monensin; in the presence of 2.1 nM PG $F_{2\alpha}$, maximal inhibition by ouabain was seen at 50 μM rather than 100 μM. This dose of prostaglandin also augmented the inhibition by a submaximal dose of monensin (0.1 μM), increasing inhibition from 5.3 ± 4.4 to 100%. Combinations of maximal doses of prostaglandin and ouabain or monensin proved to be less inhibitory than either agent alone. At 210 nM PG $F_{2\alpha}$ and 100 μM ouabain,

inhibition was 43.6 ± 4.6%; inhibition with this concentration of prostaglandin and 0.2 μM monensin was 77.2 ± 4.8%.

The augmentation of inhibition by ouabain and monensin with PG $F_{2\alpha}$ raised the question of whether inhibition by prostaglandin shared a common pharmacologic mechanism with ouabain or monensin. In particular, it was possible that PG $F_{2\alpha}$ exerted its antigonadotropic effect by increasing intracellular sodium, either by opening specific sodium channels or by inhibition of the Na^+, K^+-ATPase. To test this, cells were incubated in media in which sodium was replaced isotonically with choline chloride. LH (100 ng/ml) was added followed directly by various concentrations of PG $F_{2\alpha}$ and the incubation continued for 90 min. As seen in Figure 16, the dose-response characteristics of PG $F_{2\alpha}$ were not significantly affected by reduction of sodium levels to as low as 10 meq/l. In sodium-replete medium, 210 nM prostaglandin inhibited LH-stimulated cAMP accumulation to 33 ± 8.3% of control values; in low sodium medium, cAMP was reduced to 24.6 ± 6.3% of control values.

DISCUSSION

The present studies provide evidence that the response of the rat luteal cell to luteinizing hormone is extremely sensitive to changes in transmembrane ionic gradients. Three drugs which would be expected to perturb the normal gradients produce significant, dose-dependent inhibition of LH-stimulated cAMP and progesterone accumulation. The drugs possess different pharmacologic properties and would therefore be expected to influence the physiology of the cell in different ways. Ouabain, a cardiac glycoside, inhibits the activity of the Na^+, K^+-ATPase (54); sodium would be expected to diffuse into and potassium out of the cell according to their electrochemical gradients in absence of pump activity. Thus, both gradients would be substantially depleted, and the membrane potential decreased (54).

Monensin, a polyether carboxylic acid, is an ionophore with high specificity for sodium ions. Th ionophore also possesses some affinity for potassium, transporting K^+ about 10% as well as Na^+; monensin is also capable of transporting protons (48). Thus, the net result of treatment with monensin would be to allow sodium into the cell in exchange for potassium and protons. The resultant equilibrium levels of ionic concentrations and membrane potential would be dependent on the relative rates of ionophorous transport and pump activity of the Na^+, K^+-ATPase. Monensin has been shown to increase Na^+ uptake in neuroblastoma-glioma hybrid cells (59). Valinomycin, on the other hand, is a neutral ionophore specific for K^+; as such, valinomycin would be expected to allow potassium to diffuse out of the cell. Since the valinomycin-K^+ complex is charged, the extent of potassium diffusion is limited by the Nernst potential for K^+, and valinomycin treatment should hyperpolarize the cell membrane (48).

Each of the three drugs inhibited LH-stimulated cAMP and progesterone accumulation in a significant, dose-dependent fashion. The ID 50 for each drug was consistent with or lower than doses used to effect changes in other systems. These doses were 50 μM for ouabain, less than 1 μM for monensin, and less than 1 nM for valinomycin. Maximum inhibition was greater than 50% for each drug. None of the drugs had a consistent effect on unstimulated levels of cAMP or progesterone, although occasional slight stimulation or inhibition was seen. The lack of effect of drug treatment on trypan blue exclusion or LDH release indicates that the inhibition was not due to decreased cell viability.

The studies on the reversibility of the effect of these three drugs yielded somewhat inconclusive results. Washing decreased ouabain inhibition of cAMP accumulation from 67 to 37% although progesterone inhibition was reduced from 75 to 17%. The dissociation of the rat ouabain-ATPase complex is slow, with a $t_{1/2}$ of 5 min. in the absence of physiological ion concentrations; with the addition of monovalent cations, the $t_{1/2}$ is increased 5-fold (60). The relatively brief washings used in this study (5 min.) were most likely insufficient to allow complete dissociation of the ouabain-ATPase complex. The effect of monensin, on the other hand, was more readily reversible. Inhibition of cAMP was reduced from 68 to 2%, and progesterone inhibition was quantitatively reversed by the washing procedure. Washing paradoxically increased the inhibitory potency of valinomycin with regard to cAMP accumulation. Although this drug's lipophilic nature might make simple washing inadequate to remove the drug from cell membranes, the inhibition of progesterone accumulation did decrease from 83 to 20% by

washing. Thus, the increase in inhibitory potency for cAMP raises the questions of whether valinomycin treatment allows some essential component or cofactor of the adenylate cyclase system to leave the cell upon washing and centrifugation. Recent studies have shown that under certain circumstances, cAMP production in luteal cells is limited by the availability of purines for ATP synthesis (40). However, treatment with adenosine does not reverse valinomycin inhibition making this an unlikely explanation (Gore and Behrman, unpublished observations).

It was somewhat surprising that these drugs displayed similar dose-response curves for inhibition of cAMP and progesterone accumulation. There is a dissociation in dose-response characteristics of LH-stimulation of cAMP and progesterone secretion in luteal cells so that progesterone is maximally stimulated at doses of LH which produce very small increments in cAMP (11,36). Thus, a 50% inhibition of cAMP accumulation would not be expected to be associated with a 50% inhibition of progesterone secretion as seen with these three drugs. The experiments in which $(Bu)_2$cAMP was used to stimulate progesterone secretion revealed that ouabain and monensin inhibit progesterone secretion at a step distal to cAMP production in addition to their effects on cAMP accumulation. The reason for parallel inhibition by valinomycin remains unexplained since valinomycin did not significantly affect progesterone production stimulated by exogenous cyclic nucleotide. It is possible that valinomycin treatment specifically inhibits adenylate cyclase moities which may be directly linked to steroidogenesis. That steroidogenic and nonsteroidogenic cyclases might exist is suggested by the fact that forskolin stimulates luteal cAMP accumulation while having little effect on progesterone accumulation (Figure 11).

Because of the variety of changes in cell physiology which might result from manipulation of ionic gradients, it is impossible to assign a priori pharmacologic mechanisms responsible for the antigonadotropic effect of the drugs. Replacement of the sodium in the medium by choline demonstrated that the inhibition produced by ouabain and monensin was absolutely dependent on the presence of extracellular sodium (Figure 8). This indicates that in the case of ouabain, inhibition is not due merely to blockade of the ATPase, but rather to the resultant ionic changes, in particular, the influx of sodium according to its electrochemical gradient. Similarly, the sodium dependence of monensin inhibition is consistent with the drug's high selectivity for sodium over other cations (48) and again indicates that increasing intracellular levels of sodium antagonizes the effect of LH. This may be a direct effect of the sodium, or may be a consequence of some other change in cellular physiology triggered by the increase in sodium concentration. The potassium ionophore, as expected, did not depend on extracellular sodium for its inhibitory action, although the inhibition by valinomycin was decreased to a small extent by sodium depletion. The mechanisms by which increased intracellular sodium might antagonize LH-stimulation are discussed below.

Binding and uptake of hormone by intact cells was not affected by drug treatment. Binding studies with intact cells would appear more appropriate than membrane binding studies as the ability of these drugs to produce changes in ionic gradients depends upon the maintainance of membrane integrity with preservation of sidedness. Moreover, in preliminary studies, no inhibition of binding of [^{125}I]-hCG to luteal membranes was seen in the presence of drug treatment (data not shown). Neither ouabain nor monensin inhibited basal or

LH-stimulated cAMP production in membrane preparations; hence, these drugs do not interact directly with the adenylate cyclase complex. However, one cannot conclude that in intact cells, the changes in ionic composition do not affect the N subunit or catalytic moiety of adenylate cyclase directly. Increasing the activity of cyclic nucleotide phosphodiesterase was considered not to be a major action of these drugs as inhibition of this enzyme by IBMX did not reverse the effect of drug treatment on cAMP accumulation.

The loci of inhibition of cAMP accumulation by ouabain and monensin were defined more precisely by stimulating cells with cholera toxin and forskolin. Ouabain had no significant effect on cAMP accumulation in response to cholera toxin. Monensin, however, did reduce maximal cAMP levels by greater than 50% but had no effect on the ED 50 for cholera toxin-stimulation. As noted above, cholera toxin activates adenylate cyclase through covalent modification of N (13). Because cholera toxin-stimulated cAMP accumulation is not affected by ouabain, ouabain inhibition is most likely exerted at a step proximal to activation of N. Moreover, because ouabain has no effect on LH binding and uptake, it would appear that the glycoside inhibits functional coupling of the occupied hormone-receptor complex to adenylate cyclase. In this regard, ouabain inhibition resembles the action of PG $F_{2\alpha}$ which decreases LH-stimulated cAMP production but has little effect on cholera toxin-stimulated cAMP levels (30). The ability of monensin to inhibit cholera toxin-stimulated cAMP accumulation indicates that the inhibition by this drug may be elicited at N or a site distal to activation of N. The possibility that monensin inhibits the binding of the toxin or subsequent modification of N by the toxin cannot be excluded by the present experiments. Inhibition of

cholera toxin-stimulated cAMP production does not preclude an additional influence of monensin at the level of receptor-N interaction.

The diterpene forskolin activates adenylate cyclase in all mammalian cell types tested. Forskolin enhances ligand-stimulated cylcase activity and also markedly activates cyclase in the absence of ligand (55). Because forskolin elevates cAMP levels in the cyc$^-$ mutant S49 lymphoma cells which lack a functional N subunit, the drug is presumed to directly stimulate the catalytic subunit of adenylate cyclase (56). It is not surprising that forskolin stimulates cAMP accumulation in isolated luteal cells (Figure 10). Forskolin stimulated cAMP accumulation 5-fold. Although the maximum stimulation and ED 50 cannot be precisely determined from the current data, the dose range at which forskolin was effective (10-100 μM) is consistent with its use in other systems (55).

Ouabain had no effect on forskolin-stimulated cAMP accumulation (Figure 12A). This finding is consistent with the lack of effect of the glycoside on cholera toxin-stimulated cAMP accumulation. On the other hand, monensin inhibited forskolin-stimulated cAMP accumulation by 50% (Figure 12B). Monensin treatment would therefore appear to inhibit cAMP accumulation by changes in the sodium gradient which directly influence the catalytic moiety of adenylate cyclase. This finding is consistent with inhibition of cholera toxin-stimulated cAMP accumulation; however, it does not preclude additional effects of monensin treatment at points proximal to the catalytic subunit. Although both ouabain and monensin require extracellular sodium and calcium to express thier inhibitory activities, the experiments with cholera toxin and forskolin illustrate that a difference in the inhibitory activity of the two

drugs exists. This discrepancy may reflect differences in the extent to which the two drugs increase intracellular Na^{+} or Ca^{++} levels and the dose-response characteristics of the cations themselves.

The effect of PG $F_{2\alpha}$ on forskolin-stimulated cAMP levels was also tested. Like ouabain, PG $F_{2\alpha}$ had no effect on the binding and uptake of hCG, and had little or no effect on cholera toxin-stimulated cAMP accumulation (28,30). PG $F_{2\alpha}$ also did not inhibit forskolin stimulation of cAMP accumulation (Figure 12B), lending further support to the conclusion that PG $F_{2\alpha}$ appears to functionally uncouple the LH-receptor complex from adenylate cylase (61,71).

The inability of forskolin to substantially increase progesterone production at doses which lead to cAMP levels otherwise associated with maximal progesterone secretion (Figure 11) raises important questions about the functional relationship between cAMP levels and steroidogenesis in the luteal cell. Like forskolin, cholera toxin stimulates luteal steroidogenesis only slightly while markedly elevating cAMP to levels which, if induced by LH, would maximally stimulate progesterone production (30). Similar results have been seen with stimulation of rat leydig cells by cholera toxin (62). As discussed in the introduction, in luteal, adrenal cortical and other cells, the dose of trophic hormone required to produce half-maximal end response (in this case, progesterone accumulation) is considerably less than that which elicits the half-maximal cAMP response (11,12,36). Long attributed to "spare receptors", the additional cAMP made at high hormone concentrations may serve other cellular functions (12). The failure of forskolin and cholera toxin to stimulate progesterone secretion raises the further question of whether

different classes of adenylate cyclases exist such that the cAMP produced in response to LH gains priveleged access to the steroidogenic processes. Non-sepcific stimulation of many different cyclase complexes by cholera toxin or forskolin would lead to high levels of cAMP but only some of this would be directed to protein kinases which stimulate steroid production. Abramowitz and Birnbaumer have concluded that there is only one class of adenylate cyclase in the luteal membrane because the stimulatory effects of LH and β-adrenergic agents are not additive (14). These studies were carried out in membrane preparations and therefore cAMP production could not be correlated with steroidogenesis; it is possible that tissue homogenization and membrane isolation so disrupts the cellular architecture that in situ functional differences among cyclase molecules cannot be discerned. Further elucidation of the relationship between cAMP and steroidogenesis awaits new methodology which can resolve differences among intracellular pools of cAMP and demonstrate the presence or absence of selective stimulation and inhibition of these pools.

There are several routes by which an increase in intracellular sodium could lead to antagonism of LH-stimulation. Inhibition could be mediated directly by an action of sodium on the adenylate cyclase enzyme complex. Sodium ions reduce receptor binding affinities for cholecystokinin in brain and pancreas (63) and α-adrenergic, muscarinic, and opiate agonists in brain (64,65,66). However, ouabain and monensin had no effect on hCG binding and uptake in luteal cells. Sodium ions also promote hormonal inhibition of adenylate cyclase in various tissues including myocardium and neuroblastoma-glioma cells (67). It has been proposed that there is an

inhibitory N subunit which is linked to adenylate cyclase; the ability of increased intracellular sodium to antagonize LH-stimulated cAMP production could be mediated through activation of such a protein (67). Birnbaumer et al have reported that luteal adenylate cyclase activity in membranes is inhibited by sodium but only at doses at which non-specific effects of ionic strength are seen (10). However, it is possible that if "sided" membrane vesicle preparations were made, an increase in $[Na^+]_{in}$ might prove to specifically inhibit LH-stimulated cAMP production.

LH-stimulated cAMP levels were markedly reduced when ouabain or monensin were added simultaneous with or preceeding LH (Table 4). In the case of ouabain, addition of the drug before or together with LH led to 48% inhibition, whereas addition 15 min. following the hormone led to only 12% inhibition. The binding of ouabain to the Na^+, K^+-ATPase is slow, with a $t_{1/2}$ of 10 min. (68). Additionally, some time must be required for the subsequent passive depletion of sodium and potassium gradients. Thus, when ouabain is added after LH, considerable amounts of cAMP might accumulate before ouabain takes effect. Alternatively, LH pretreatment may render the cell resistant to inhibition by ouabain. Monensin inhibition of cAMP accumulation was also considerably diminished when the drug was added after LH. Monensin action requires diffusion of the drug into the membrane, after which the ionophore acts as a pore, allowing sodium to diffuse into the cell. Thus, monensin may not require as long a latent period for activity as ouabain. It seems likely that LH pretreatment has a protective effect against monensin inhibition. In contrast, PG $F_{2\alpha}$ effectively antagonizes LH action even when added 60 min. after LH (57). In this respect, ouabain and monensin

inhibition differ from that of prostaglandin. The binding of hCG to LH receptors is essentially irreversible at 37°C (69). It is tempting to postulate that coupling of occupied LH receptors to cyclase is also not easily reversed, a phenomenon which may diminish ouabain and monensin action after LH addition. It is currently thought that hydrolysis of GTP by the N subunit inactivates ligand-stimulated adenylate cyclase while decreasing the affinity of the receptor for ligand (70). However, GTP does not change the affinity of the LH receptor for gonadotropin as it does for ligand receptors in other cyclase systems (71,72,73); LH-stimulated adenylte cyclase may be inactivated by other means, such as internalization of the hormone-receptor complex.

Increasing intracellular sodium levels depolarizes the cell membrane. It is conceivable that a negative membrane potential is needed for effective stimulation of adenylate cyclase by LH. For example, if maximal stimulation by LH required an acute influx of sodium or calcium through receptor-mediated channels, chronic depolarization might inactivate such channels. This seems unlikely as Higuchi and colleagues showed through direct intracellular recording of luteal cell membrane potential that LH did not change membrane potential (46). In these studies, depolarization by high concentrations of potassium actually increased progesterone secretion; cAMP was not measured. Recently, however, adrenocortical cells have been demonstrated to undergo depolarization in response to ACTH; this depolarization appears to be due primarily to calcium influx (74).

If an acute ionic flux or depolarization were required for full activation of adenylate cyclase by LH, this might explain the diminished effect of ouabain and monensin following LH addition as these drugs induce chronic

depolarization. However, substantial reduction of the sodium gradient by decreasing extracellular sodium to 32 meq/l had no effect on LH stimulation (Figure 13). The sodium channel blocker tetrodotoxin also had no effect on LH-stimulation. This does not exclude a role for sodium influx in LH-stimulation as TTX appears to block potential-dependent sodium gates; receptor-mediated sodium permeability changes such as in post-synaptic membranes are not blocked by TTX (75,76). However, depolarization of luteal cells by high extracellular potassium concentrations had no effect on LH-stimulated cAMP accumulation (Figure 14). Since sodium channels would be expected to be inactivated by chronic depolarization, a role of acute ion flux in LH-stimulation is unlikely. The present results also show that depolarization does not increase luteal cAMP accumulation (Figure 14). As depolarization appears to increase luteal steroidogenesis (46), this may indicate that there are additional factors regulating luteal steroidogenesis which mediate depolarization-induced progesterone accumulation. There is ample evidence in the adrenal cortex that calcium is required for steroid biosynthesis; both depolarization and ACTH stimulate calcium influx (12).

Increasing intracellular sodium often leads to a secondary increase in intracellular calcium (77). There is considerable debate as to whether this results from an increase in sodium-calcium antiport, inhibition by intracellular sodium of calcium extrusion, or a decrease in intracellular calcium sequestration; it is likely that in various cell types and under different conditions each mechanism may play a role (58). It is generally agreed that the inotropic action of ouabain results from a secondary increase in intracellular calcium (54). Calcium ions could be responsible for

inhibiting LH-stimulated cAMP production. Dorflinger and Behrman reported that two hour incubations of luteal cells with the calcium ionophore A23187 antagonized the effects of LH; this inhibition required the presence of extracellular calcium (31). Ouabain decreases thyrotropin-stimulated cAMP accumulation in canine thyroid slices; this effect was partially reversed by Mn^{++} ions, but was not dependent on the presence of extracellular calcium (78). The authors suggested that the inhibition was mediated by decreased sequestration of intracellular calcium or release of calcium from organelles in response to the increased intracellular sodium.

Current experiments in which the concentration of extracellular calcium was varied point to a complicated role for this cation in luteal physiology (Table 5). LH stimulates cAMP production in the absence of extracellular calcium; replacing calcium increases both stimulated and basal cAMP levels. Maximum cAMP levels in response to LH were seen at 1.5 mM calcium. Extracellular calcium thus appears to enhance LH-stimulation of cAMP but it does not appear to be obligatory. It is not clear from the present study whether this effect of calcium is strictly extracellular, for instance by enhancing binding of hormone, or whether LH-stimulation actually depends on a calcium influx. Extracellular calcium has similar effects on cAMP production in adrenal cells stimulated by ACTH (79,80) and C-6 glioma cells stimulated by norepinephrine (81). However, as noted, A23187 treatment inhibits LH stimulated cAMP levels (31). It is possible that low levels of cytoplasmic calcium enhance LH-stimulated cAMP production while higher concentrations inhibit cyclase directly. Such biphasic modulation of adenylate cyclase activity by calcium has been demonstrated in brain tissue. A

calmodulin-sensitive adenylate cyclase has been shown to be stimulated by micromolar calcium concentrations and inhibited by higher concentrations of calcium (82). Calcium is not required to elicit LH-stimulation of adenylate cyclase activity in luteal membranes (10). However, luteal membranes have required superphysiological doses of LH to generate cAMP, and the absence of calcium may contribute to this (10). Millimolar concentrations of calcium inhibit LH-stimulated cyclase activity in membranes (31). It is possible that under appropriate circumstances, for instance with the addition of calcium and calmodulin, a direct role of calcium in physiological concentraions on LH-sensitive adenylate cyclase will be demonstrated in membrane preparations. Aside from inhibiting adenylate cyclase directly or via calmodulin, increased cytoplasmic calcium could stimulate phospholipase A_2 or C, producing prostaglandins or phosphatidyl inositol metabolites which could mediate the inhibition of adenylate cyclase. A similar mechanism could explain A23187 inhibition.

Because replacing extracellular calcium enhances LH-stimulated cAMP accumulation, the role calcium plays in mediating ouabain and monensin inhibition remains ambiguous. In the absence of extracellular calcium neither drug affects LH-stimulated cAMP production; replacement of calcium allows the inhibitory activity of the drugs to be expressed (Table 5). There are two possible interpretations for these results. One is that LH-stimulation of cAMP has two components, one calcium-dependent and one calcium-independent. If ouabain and monensin inhibit the calcium-dependent component, the inhibition by the drugs would not be observed in the absence of calcium. It is also possible and perhaps more likely that the increase in intracellular

sodium provided by ouabain and monensin treatment leads to an influx of extracellular calcium and this leads directly or indirectly to inhibition of LH-stimulated cAMP production. This would be consistent with the effects of A23187. The inhibition of LH-stimulation seen by complete removal of sodium from the medium (Figure 13) could also be explained by a rise in intracellular calcium mediated by sodium-calcium exchange induced by a reversal of the sodium gradient (77).

PG $F_{2\alpha}$ does not require extracellular calcium for its antigonadotropic activity (57). The effects of low doses of PG $F_{2\alpha}$ and ouabain or monensin are superadditive, although combinations of high doses of prostaglandin with either drug are less inhibitory than either treatment alone (Figure 15). This additivity at low doses may indicate that ouabain and monensin share a common mechanism of inhibition with PG $F_{2\alpha}$. However, PG $F_{2\alpha}$ was equally potent in the presence and absence of extracellular sodium (Figure 16). It is thus highly unlikely that PG $F_{2\alpha}$ increases intracellular sodium as a primary mechanism of inhibition. PG $F_{2\alpha}$ may, however, release calcium from intracellular stores. If inhibition by ouabain and monensin are mediated by calcium, additivity would be predicted. It is also possible that some or all of the inhibition produced by ouabain and monensin is due to synthesis of prostaglandin. There are differences between the inhibitory activities; PG $F_{2\alpha}$ is active whether added before or after LH (57), whereas ouabain and monensin are less active when added after LH. Monensin appears to lead to inhibition of the catalytic subunit of adenylate cyclase, while PG $F_{2\alpha}$ and ouabain appear to uncouple the hormone-receptor complex from N. If ouabain and monensin activity were due exclusively to synthesis of prostaglandin, one would expect activity but not superadditivity with low doses of PG $F_{2\alpha}$.

Ouabain treatment in different cell types affects cyclic nucleotide levels in various ways. As noted above, in thyroid tissue, ouabain decreases TSH-stimulated cAMP accumulation (78). In cultured fetal rat bones, ouabain increased basal and PTH-stimulated cAMP levels (83) but had no effect on PG $F_{2\alpha}$-stimulated cAMP accumulation in cultured bone cells (84). Ouabain also increases cAMP levels and insulin secretion in isolated pancreatic islet cells (85). Monensin stimulates catecholamine secretion in adrenal chromaffin cells (86) and in pheochromocytoma cells (87) and increases spontaneous and evoked transmitter release in the frog neuromuscular junction (88). In both adrenal and pheochromocytoma cells, the effect of monensin required extracellular sodium but not calcium. There have been few reports of the effects of monensin on cAMP levels. In sea urchin spermatozoa, high concentrations of the ionophore (25 μM) stimulate cAMP accumulation in a sodium-dependent fashion (89).

The mechanism of the antigonadotropic effect of valinomycin is not obvious. Valinomycin treatment should result in hperpolarization of the membrane as noted above; this would increase the electrochemical gradient for both sodium and calcium. Valinomycin inhibition is only slightly dependent on extracellular sodium (Figure 8). Preliminary experiments indicate that inhibition by valinomycin is, however, dependent on the presence of extracellular calcium (data not shown). This lends support to the possibility that a primary step in inhibition of LH-stimulated cAMP accumulation by all three drugs in an increase in intracellular calcium.

It seems likely that increased intracellular calcium, produced by a variety of pharmacologic manipulations, inhibits the response of the luteal

cell to LH in agreement with earlier findings using the calcium ionophore A23187 (31). Direct confirmation of this hypothesis will require the use of labeled calcium to study calcium flux in response to drug treatment. Inhibition by calcium might be mediated directly at the adenylate cyclase complex as suggested by preliminary studies which showed inhibition of LH-stimulated cAMP in membrane preparations by millimolar levels of calcium (31). Alternatively, calcium influx could lead to the activation of phospholipases whose reaction products-prostaglandins, leukotrienes or phosphatidyl inositol metabolites-antagonize LH action. Additionally the effects of calcium may be related to changes in membrane fluidity, consistent with the x-ray diffraction experiments of Carlson and colleagues (42,43,44).

Precise explication of the role of calcium in LH-activation and inhibition of LH-activation will require experiments with membrane preparations testing the effects of calcium, calmodulin, calmodulin inhibitors or antibodies, on LH-stimulated cAMP production. As LH-stimulation is blunted in the absence of extracellular calcium, it seems likely that under appropriate experimental conditions, LH-sensitive adenylate cyclase will be stimulated at low calcium concentrations, but will be inhibited by higher concentrations, similar to some cyclase systems in brain (82). Additional experiments with inhibitors of phospholipases and cyclo- and lipo- oxygenase would help determine whether production of fatty acid-derived mediators is an important consequence of calcium influx in the luteal cell.

The luteal cell is unique as it becomes desensitized to LH spontaneously during its brief lifetime. The ability of three ion-active drugs to profoundly inhibit the response of luteal cells to luteinizing hormone

indicates that maintainance of the usual ionic gradients is critically important to luteal steroidogenesis. Indeed, the competitive actions of these drugs and natural substances such as PG $F_{2\alpha}$ and GnRH against LH may indicate that one of the luteotropic actions of LH may be to maintain optimum electrochemical ionic gradients in the luteal cell and to preserve low intracellular levels of calcium. This conclusion is supported by results of the present studies which showed that ouabain and monensin were inactive in cells pretreated for about 30 min. with LH. Consequently, LH may rescue the corpus luteum from regression by preventing an increase in intracellular calcium which may be the initial event in the luteolytic process. Detaiiled investigation into the effects of calcium on the luteal adenylate cyclase complex may yield important information about luteolysis, receptor-cyclase coupling and modulation of cellular response to extracellular messengers.

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Table 1. Reversibility of inhibition of LH-stimulated cAMP and progesterone accumulation by ouabain, monensin and valinomycin.

	cAMP		Progesterone	
Treatment	Drug Treated	Drug Treated and Washed	Drug Treated	Drug Treated and Washed
	% Inhibition		% Inhibition	
Ouabain (100 μM)	67.3 ± 7.1	36.8 ± 3.7[a]	75.7 ± 2.7	17.8 ± 2.8[a]
Monensin (0.2 μM)	68.4 ± 6.3	2.2 ± 0.3[a]	100	-25.7 ± 2.9[ab]
Valinomycin (2 nM)	18.1 ± 1.5	53.3 ± 3.8[a]	82.8 ± 23.8	20.2 ± 0.8[a]

Cells were incubated with drugs at the indicated concentrations for 15 min, the media was aspirated following centrifugation (100 g; 5 min), fresh media with and without drugs was added, the cells were resuspended and incubated for an additional 5 min. The washing procedure was then repeated. The cells were resuspended in fresh media with and without drugs and incubated for 5 min, following which LH (100 ng/ml) was added and the incubation continued for an additional 90 min.

Values are the mean ± SEM of four replicates, expressed as percent inhibition of control LH stimulated. [a]Significantly different from washed and retreated ($p < 0.05$). [b]Washed values greater than control

Table 2. Effect of ouabain on adenylate cyclase activity in luteal membranes.

	cAMP (pmol/mg/min)	
[LH] (ng/ml)	Control	Ouabain (100 μM)
0	8.1 ± 1.3	6.8 ± 0.5
50	11.0 ± 1.1	14.2 ± 1.3
100	21.3 ± 4.0	21.0 ± 4.0

Values are the mean ± SEM of four replicates.

Table 3. Effect of monensin on adenylate cyclase activity in luteal membranes.

	cAMP (pmol/mg/min)	
[LH] (ng/ml)	Control	Monensin (1 μM)
0	1.0 ± 0.6	1.5 ± 1.3
50	10.2 ± 1.6	6.8 ± 1.7[a]
100	13.7 ± 1.0	13.7 ± 1.2

Values are the mean ± SEM of four replicates. [a]Difference not significant ($p > 0.05$).

Table 4. Effect of time of addition of ouabain or monensin on inhibition of LH-stimulated cAMP accumulation.

Time of addition of drug (min)	Inhibition of cAMP Accumulation (%)	
	Ouabain (100μ M)	Monensin (0.2μ M)
-60	*37.9 ± 2.4	
-30		*49.7 ± 3.8
-15	*48.0 ± 0.1	*45.0 ± 2.4
0 (LH added)	*48.7 ± 2.7	*18.5 ± 3.2
15	*12.0 ± 3.1	*19.6 ± 5.3
30	1.4 ± 5.0	*11.4 ± 1.7
60	5.5 ± 4.7	-26.7 ± 1.9[a]

Values are the mean ± SEM of four replicates expressed as percent inhibition of LH-stimulated levels (LH = 200 ng/ml for ouabain and 50 ng/ml in monensin experiment). *$p < .05$ compared to LH control. At the indicated times drug or media alone was added. [a]Value greater than LH control. All incubations with LH in the presence of drugs were of 90 min. duration.

Table 5. Effect of calcium depletion on LH-stimulation of cAMP accumulation and on inhibition of LH-stimulation by ouabain and monensin in luteal cells.

Treatment	$CaCl_2$ (mM) 0	0.5	1.5
Control	1.0 ± 0.3	1.0 ± 1.6	4.0 ± 1.0
LH (100 ng/ml)	3.7 ± 0.4	6.7 ± 0.9	14.4 ± 2.6
LH + Ouabain (100 μM)	3.8 ± 1.0	4.0 ± 0.3*	6.1 ± 1.1*
LH + Monensin (0.2 μM)	5.0 ± 1.1	8.4 ± 0.3	2.6 ± 0.3*

Cells were dispersed and enriched in Ca^{2+} -free media (see Materials and Methods) and incubated in the same media to which the indicated concentrations of $CaCl_2$ was added. Cells (10^5) were preincubated with ouabain or monensin for 20 min prior to addition of LH; drugs and LH were coincubated with the cells for 90 min. Each value is the mean ± SEM of 4 replicates. *$p < .01$ compared to LH treatment alone.

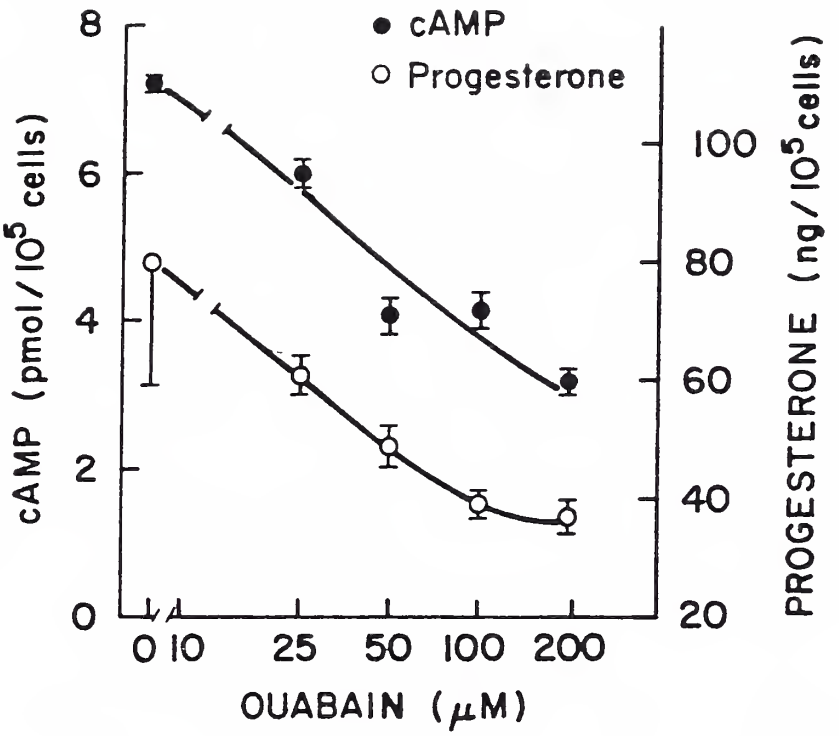

FIGURE 1. Dose-response effect of ouabain on LH stimulated cAMP and progesterone accumulation in luteal cells. Cells (10^5) were incubated with ouabain for 1 h, at which time LH was added (200 ng/ml). Incubation was continued for 90 min. Each point is the mean ± SEM for four replicates. cAMP accumulation in the absence of LH was 0.2 pmol/10^5 cells. Progesterone accumulation in the absence of LH was 21.3 ± 1.0 ng/10^5 cells.

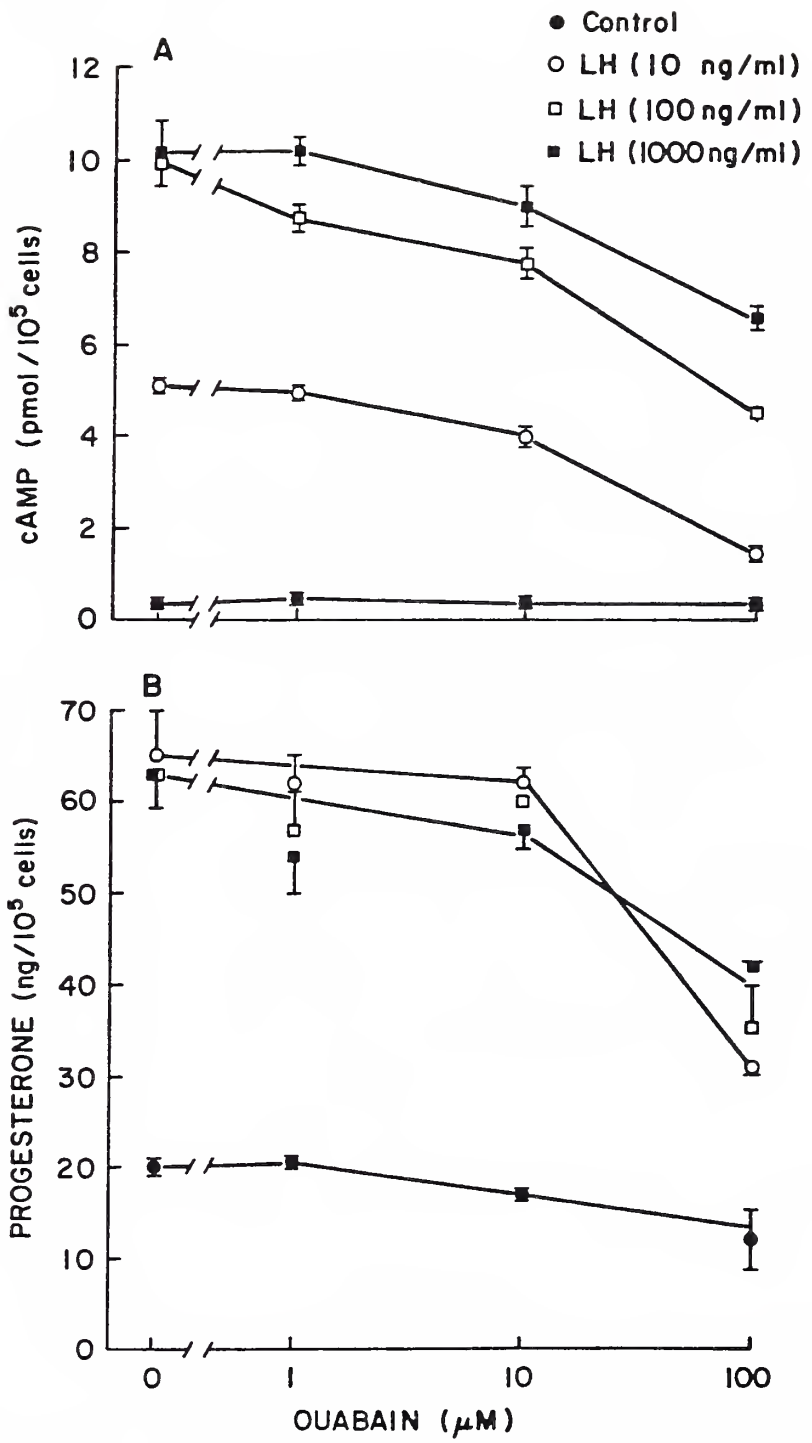

FIGURE 2. Effect of LH concentration on inhibition of cAMP and progesterone accumulation by ouabain in luteal cells. Cells (10^5) were incubated with ouabain for 1 h, at which time LH was added. Incubation was continued for 90 min. Each point is the mean ± SEM of four replicates. A. cAMP accumulation. B. Progesterone accumulation.

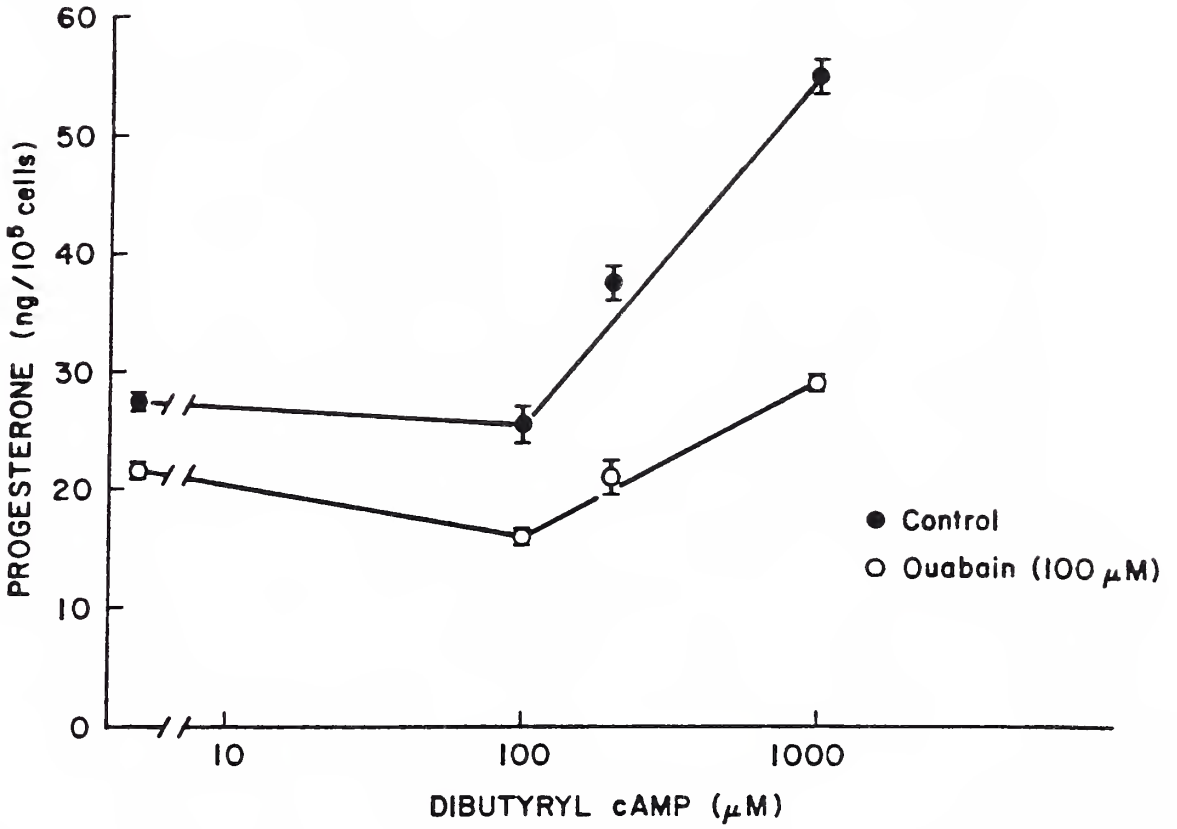

FIGURE 3. Effect of ouabain on stimulation of progesterone accumulation by $(Bu)_2$-cAMP in luteal cells. Cells (10^5) were incubated with ouabain (100 μM) for 30 min. before the addition of $(Bu)_2$-cAMP. Incubation was continued for 90 min. Each point represents the mean ± SEM of four replicates.

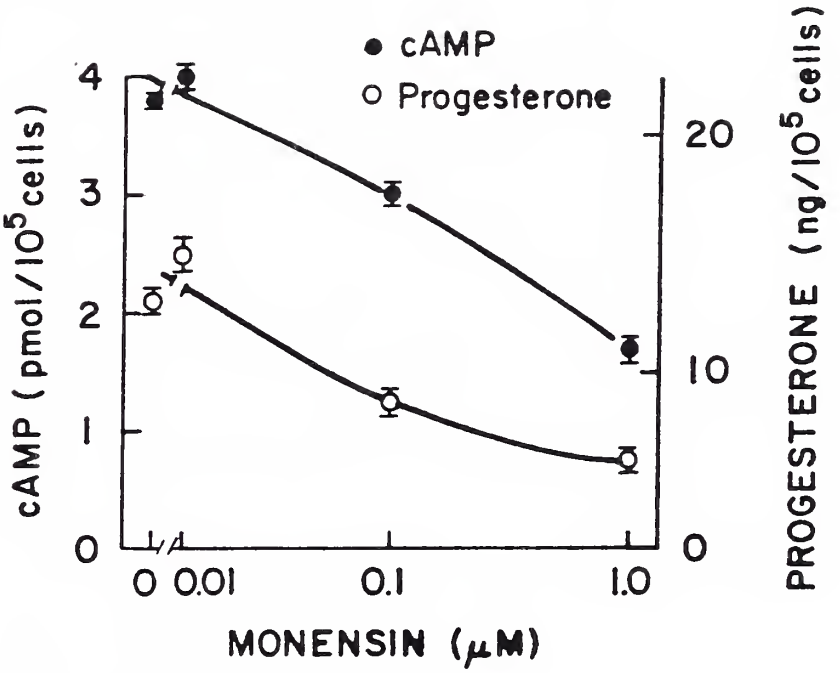

FIGURE 4. Dose-response effect of monensin on LH-stimulated cAMP and progesterone accumulation in luteal cells. Cells (10^5) were incubated with monensin for 30 min. before the addition of LH (200 ng/ml). Incubation was continued for 90 min. Each point represents the mean ± SEM of four replicates. cAMP accumulation in the absence of LH was 0.1 pmol/10^5 cells. Progesterone accumulation in the absence of LH was 3.7 ± 0.6 ng/10^5 cells.

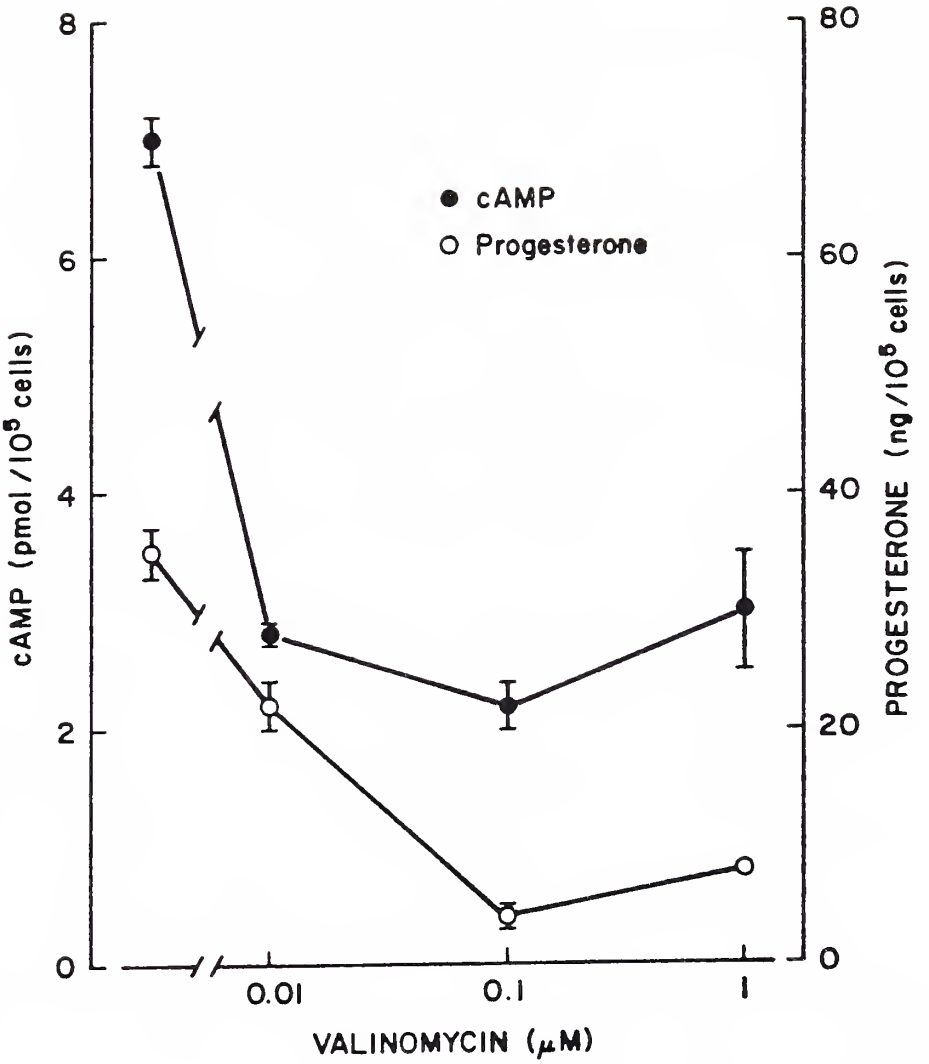

FIGURE 5. Dose response effect of valinomycin on LH-stimulated cAMP and progesterone accumulation in luteal cells. Cells (10^5) were incubated with valinomycin for 30 min. before the addition of LH (50 ng/ml). Incubation was continued for 90 min. Each point represents the mean $\pm$ SEM of four replicates. cAMP accumulation in the absence of LH was 0.2 pmol/10^5 cells. Progesterone accumulation in the absence of LH was 15.6 $\pm$ 0.4 ng/10^5 cells.

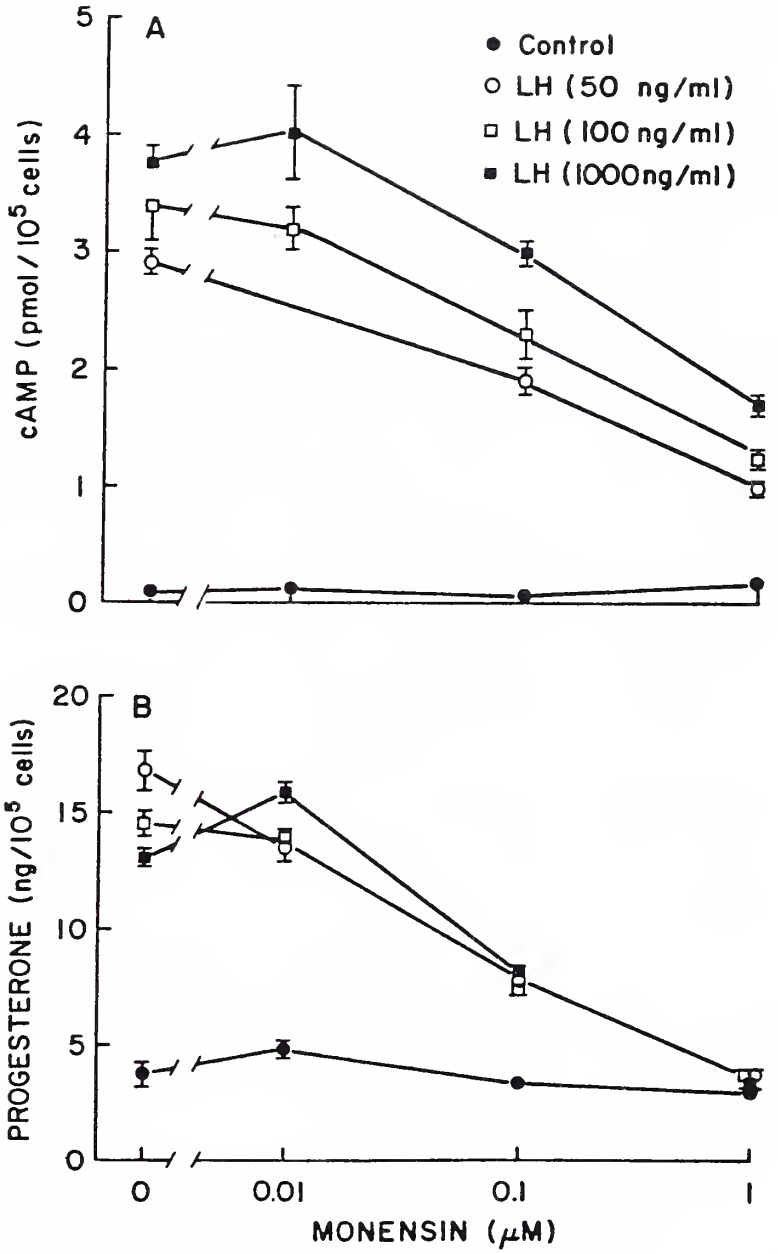

FIGURE 6. Effect of LH concentration on inhibition of cAMP and progesterone accumulation in luteal cells by monensin. Cells (10^5) were incubated with monensin for 30 min. before the addition of LH. Incubation was continued for 90 min. Each point is the mean ± SEM of four replicates. A. cAMP accumulation. B. Progesterone accumulation.

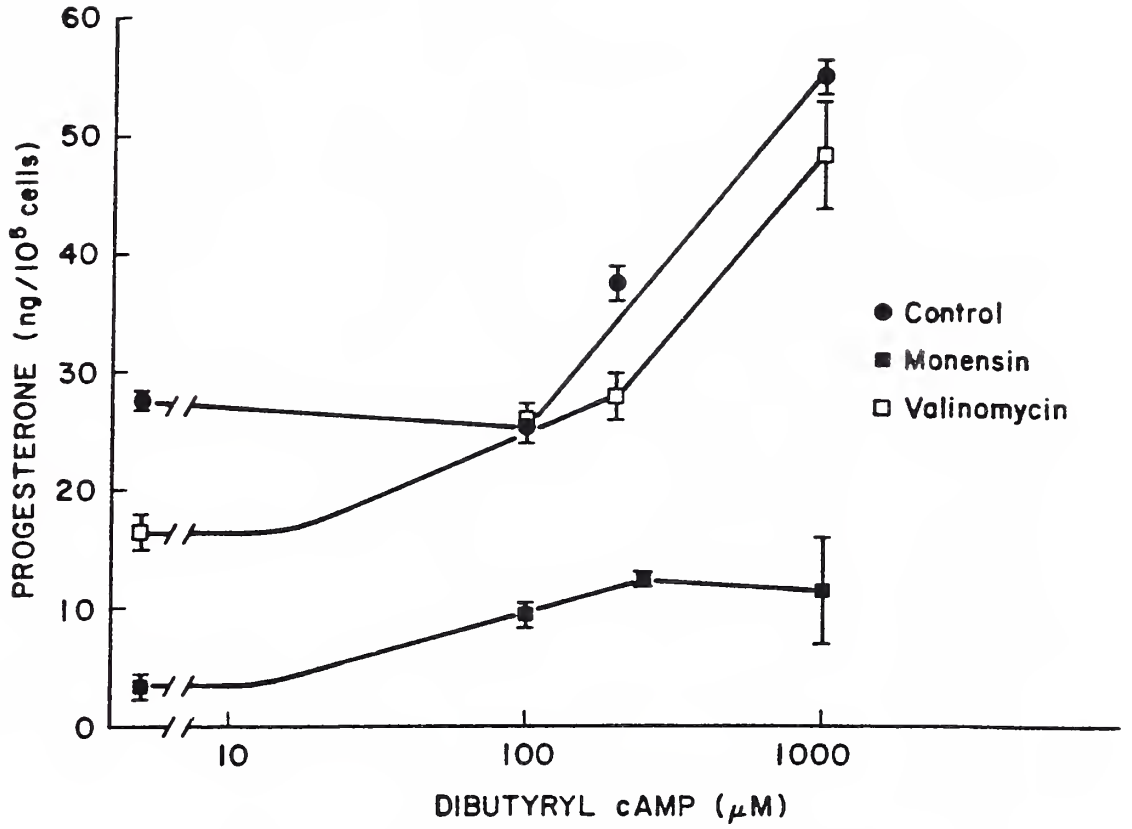

FIGURE 7. Effects of monensin and valinomycin on stimulation of progesterone accumulation by $(Bu)_2$-cAMP in luteal cells. Cells (10^5) were incubated with monensin (0.2 μM) or valinomycin (2 nM) for 30 min. before the addition of $(Bu)_2$-cAMP. Incubation was continued for 90 min. Each point represents the mean ± SEM of four replicates.

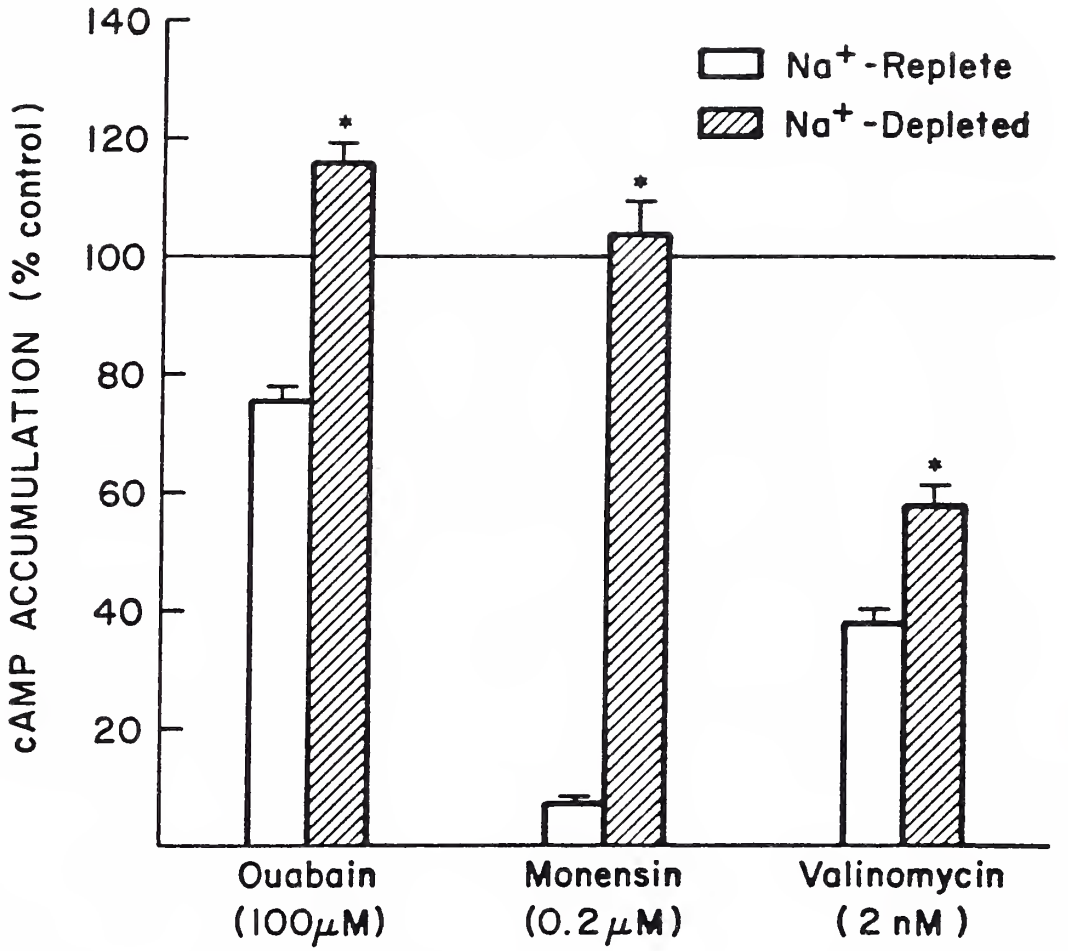

FIGURE 8. Effect of sodium depletion on inhibition of LH-stimulated cAMP accumulation by ion-active drugs in luteal cells. Cells were washed twice in sodium-replete medium or in medium in which sodium was replaced isotonically with choline chloride, as described in Methods. Cells (10^5) were incubated with ouabain (100 μM), monensin (0.2 μM) or valinomycin (2 nM) for 30 min. before the addition of LH (100 ng/ml). Each value represents the mean ± SEM for four replicates. cAMP accumulation in the absence and presence of LH was 0.07 ± 0.03 and 7.1 ± 0.3 pmol/10^5 cells, respectively, in sodium-replete medium, and 0.07 ± 0.04 and 4.2 ± 0.2 pmol/10^5 cells, respectively, in sodium-depleted medium. *significantly different from control ($p < 0.05$).

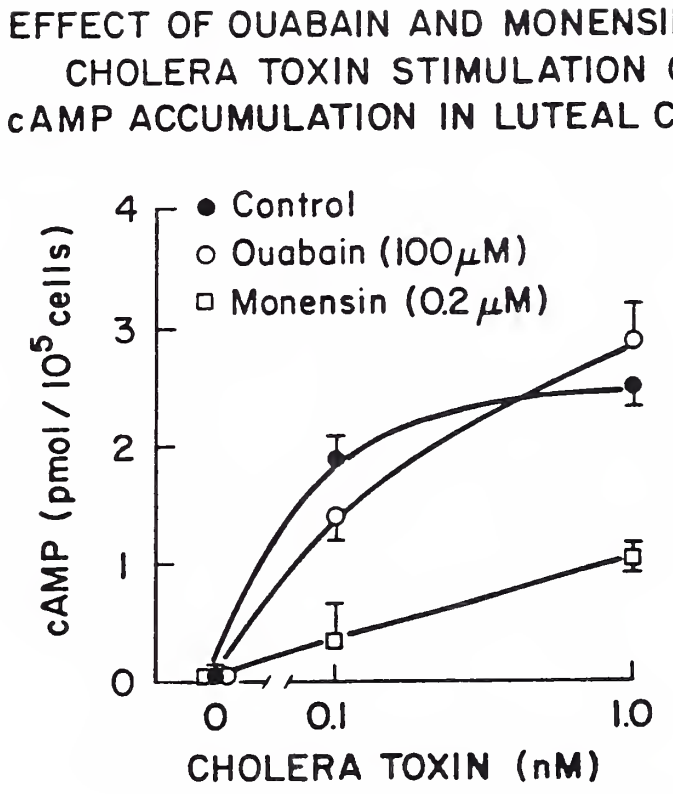

FIGURE 9. Effect of ouabain and monensin on cholera toxin-stimulated cAMP levels in luteal cells. Cells (10^5) were incubated with ouabain (100 μM) or monensin (0.2 μM) for 20 min. before the addition of cholera toxin. Incubation proceeded for 2 h. Each point represents the mean $\pm$ SEM of four replicates.

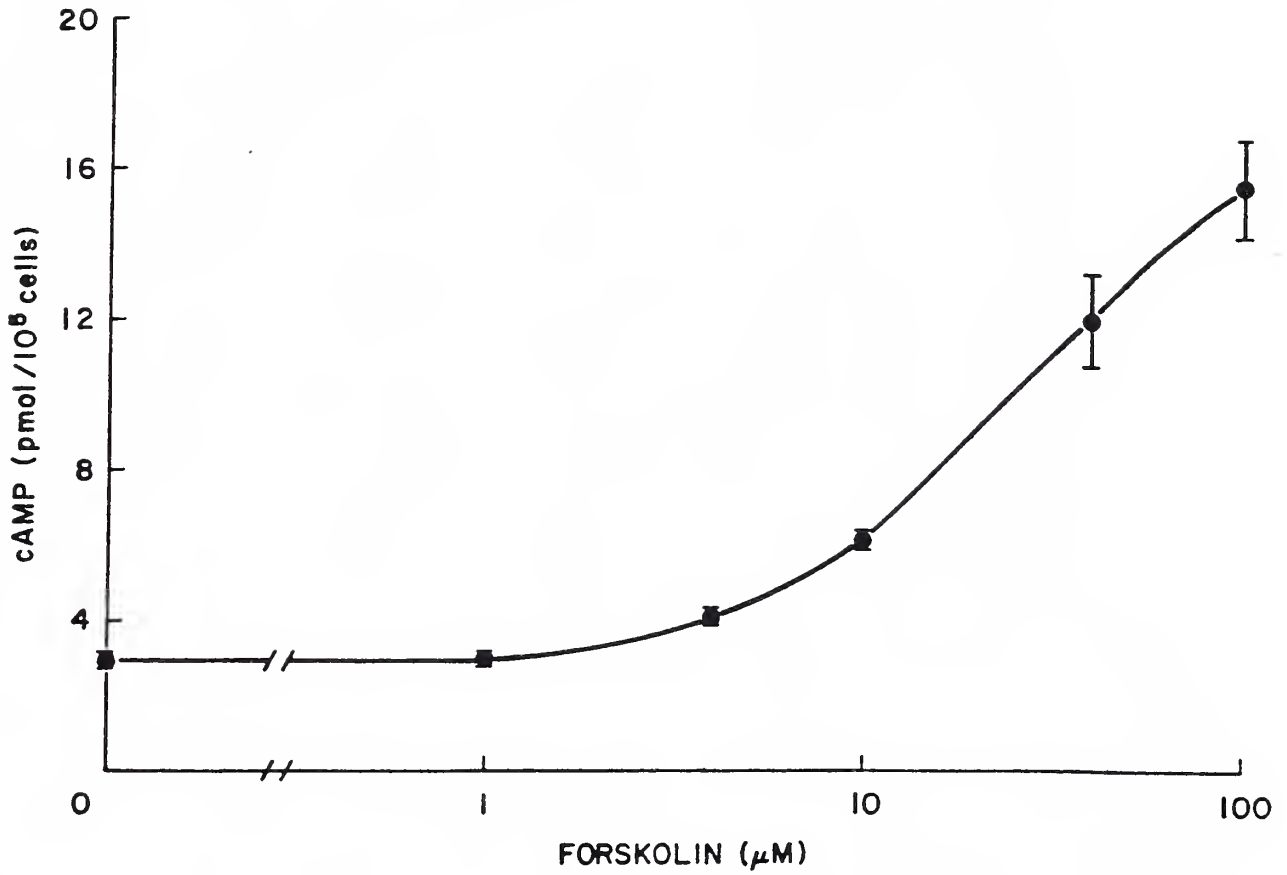

FIGURE 10. Dose-response effect of forskolin on cAMP accumulation in luteal cells. Cells (10^5) were incubated with forskolin for 1 h. Each point represents the mean ± SEM for four replicates.

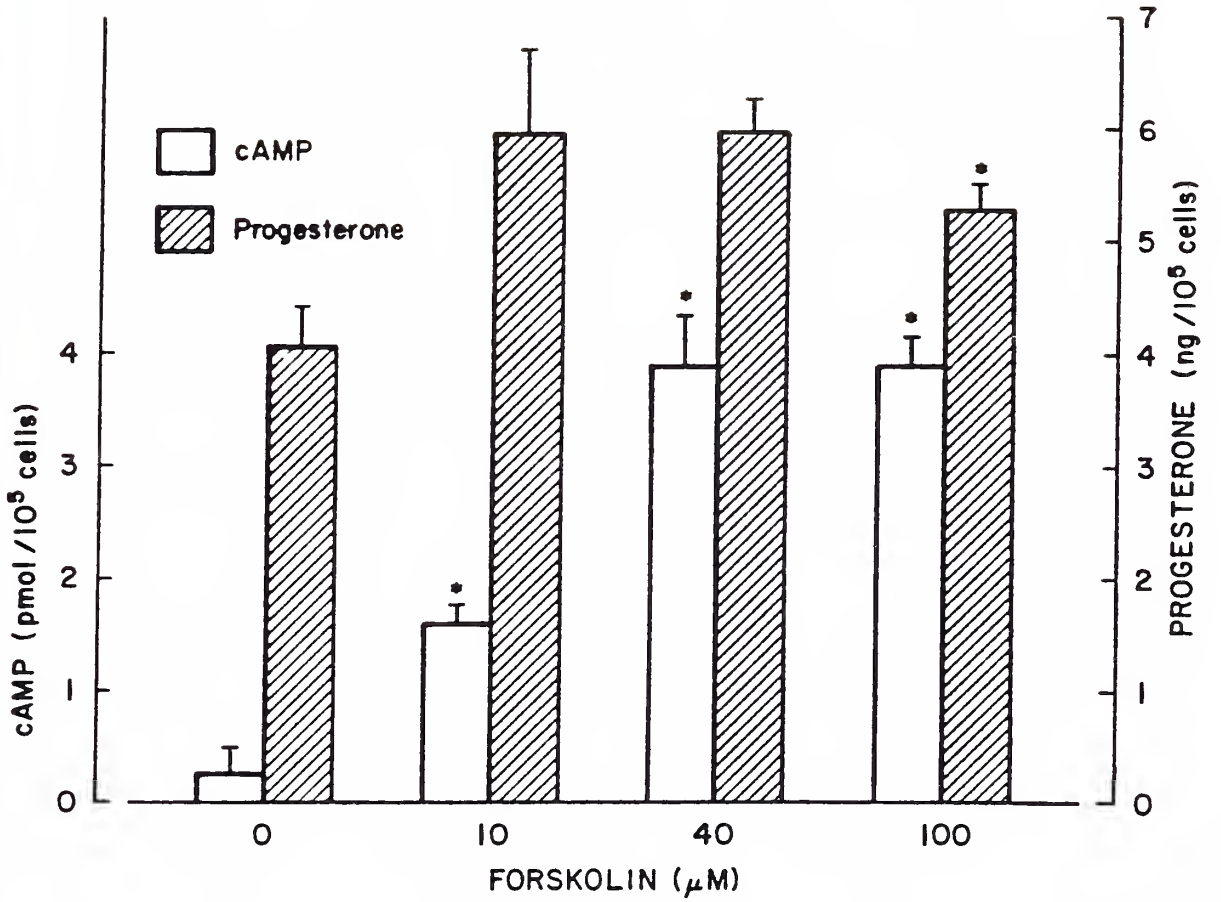

FIGURE 11. Comparison of effects on cAMP and progesterone accumulation by forskolin in luteal cells. Cells were incubated with forskolin for 1 h. Each point represents the mean ± SEM of four replicates. *significantly different from control ($p < 0.05$).

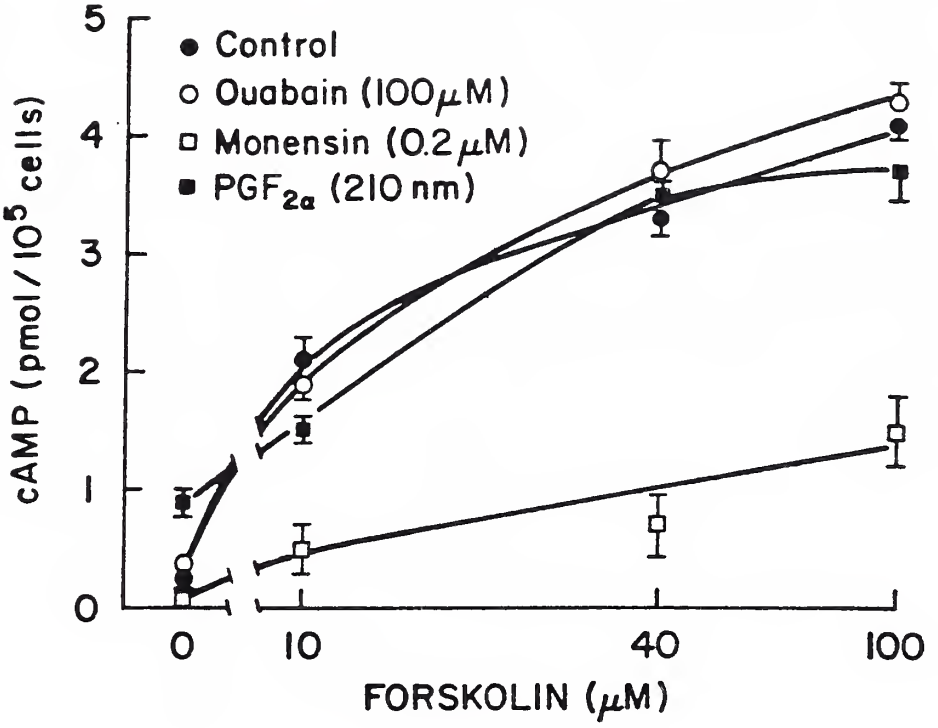

FIGURE 12. Effect of ouabain, monensin, and PG F_2 on forskolin-stimulation of cAMP accumulation in luteal cells. Cells (10^5) were incubated with ouabain (100 μM) or monensin (0.2 μM) for 20 min. before the addition of forskolin. PG $F_{2\alpha}$ (210 nM) was added simultaneously with forskolin. Incubation proceeded for 60 min. Each value represents the mean ± SEM of four replicates.

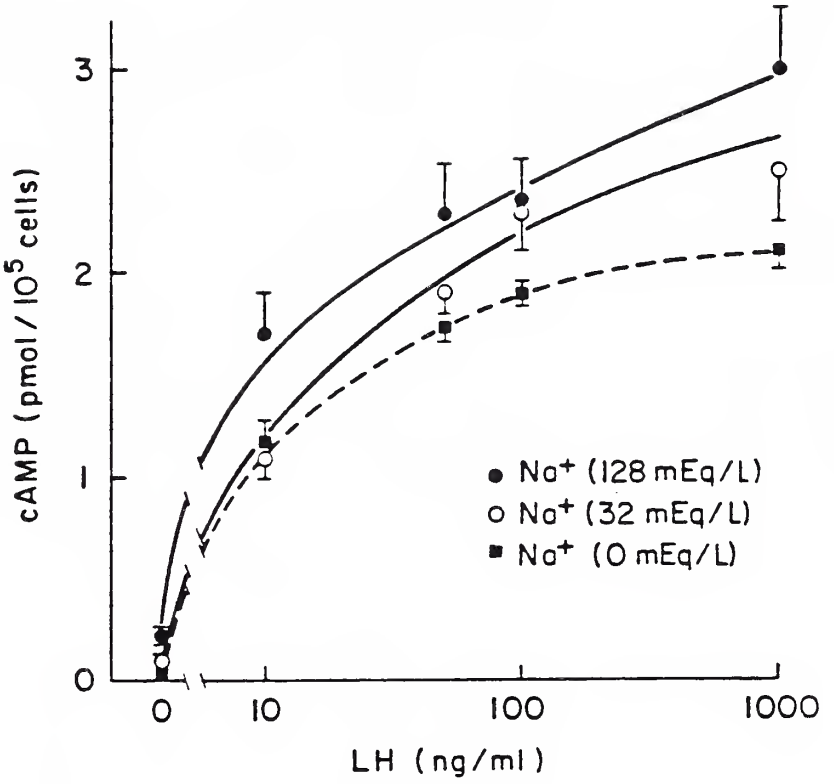

FIGURE 13. Effect of Na^+ concentration on LH-stimulated cAMP accumulation in luteal cells. Cells were washed twice with sodium-replete medium or with medium in which Na^+ was replaced isotonically with choline chloride (see Materials and Methods). Cells (10^5) were then resuspended in medium which contained the indicated concentrations of Na^+ and incubated with LH (100 ng/ml) for 90 min. Each point represents the mean $\pm$ SEM of 4 replicates.

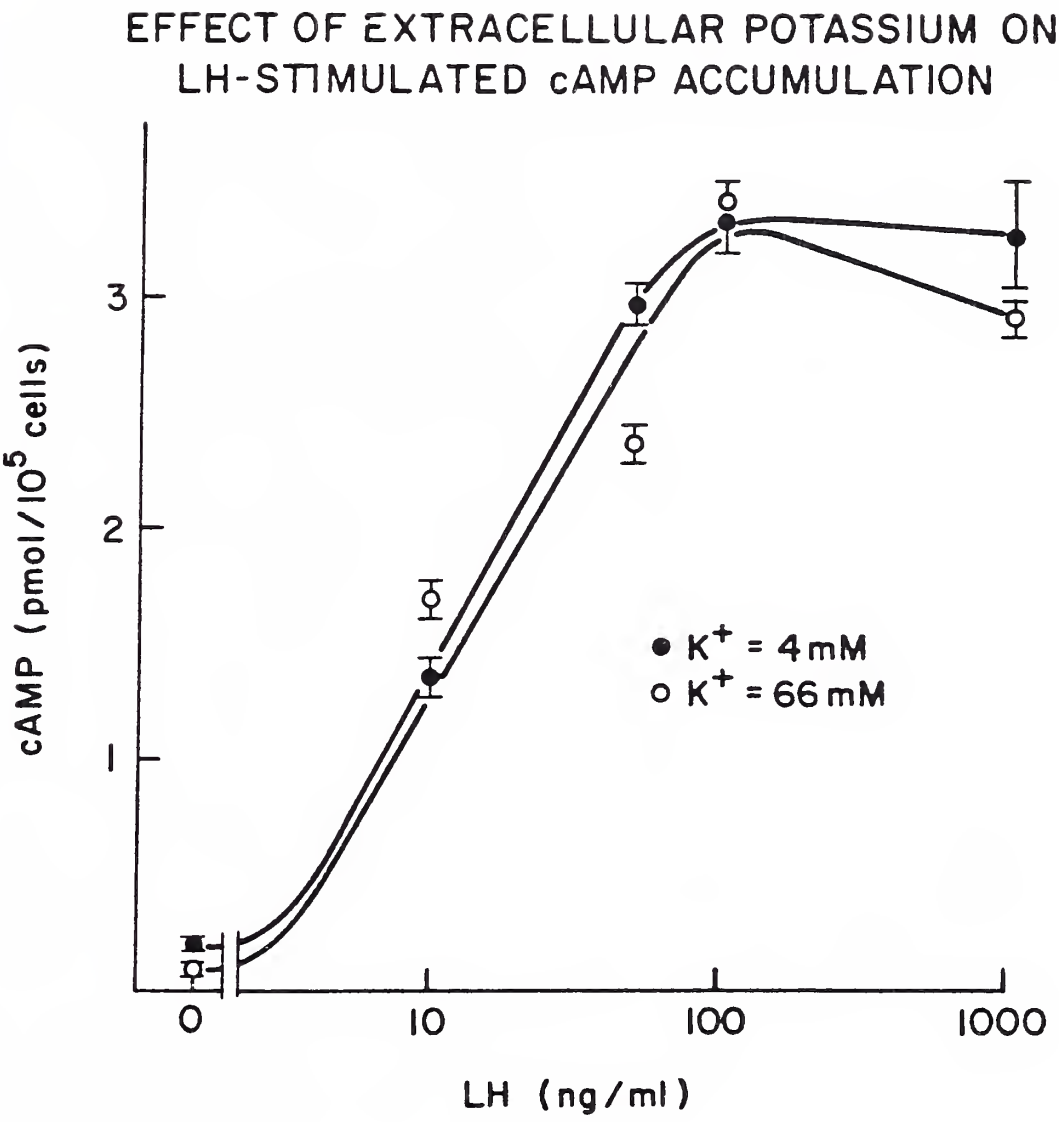

FIGURE 14. Effect of depolarization by high extracellular potassium on LH-stimulated cAMP levels in luteal cells. Cells were washed and resuspended in medium in which sodium chloride was replaced isotonically with potassium chloride. LH was added and cells were incubated for 90 min. Each point represents the mean ± SEM for four replicates.

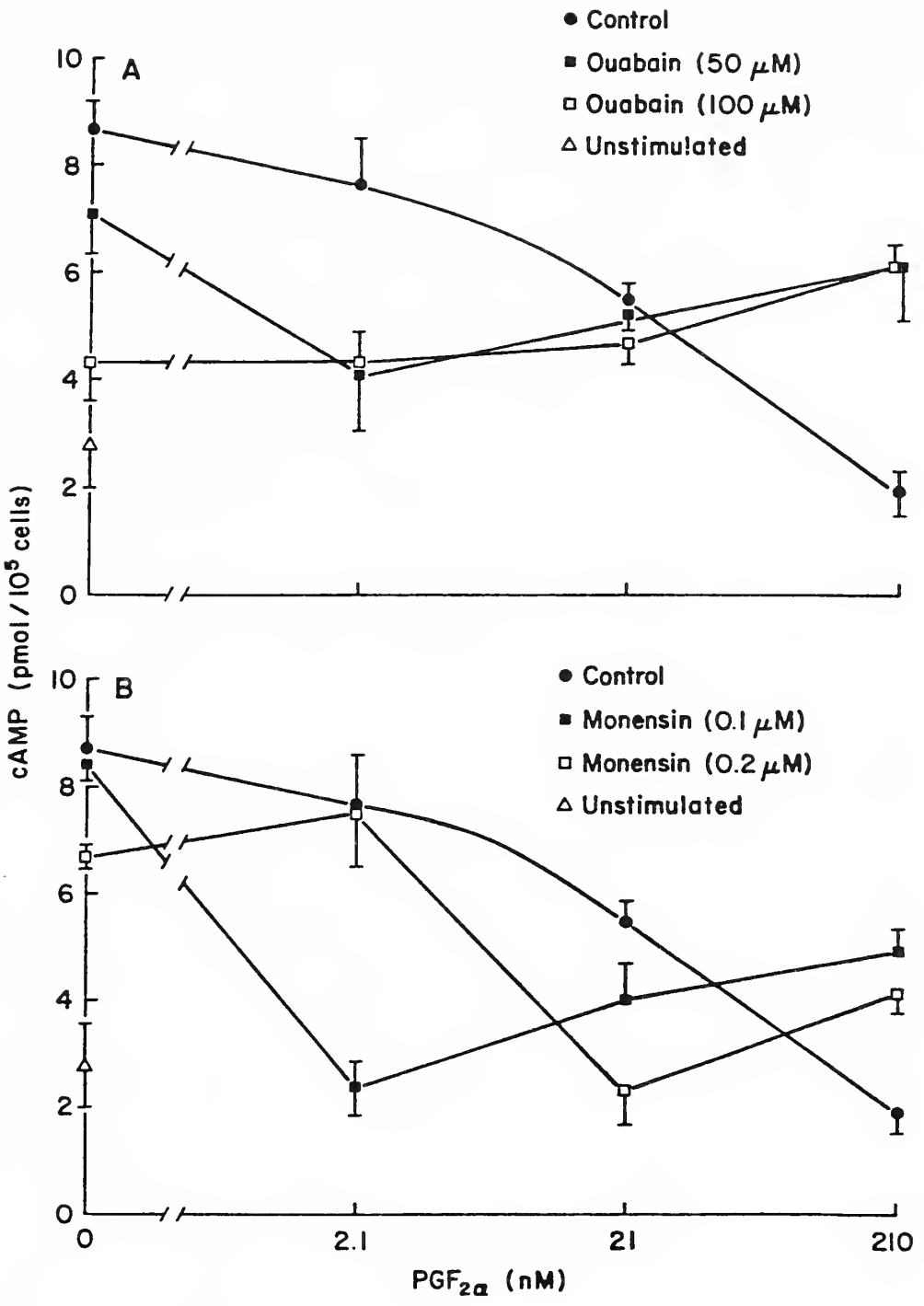

FIGURE 15. Combined effects of ouabain or monensin with PG $F_{2\alpha}$ on LH-stimulated cAMP accumulation in luteal cells. Cells (10^5) were incubated with ouabain (50-100 μM) or monensin (0.1-0.2 μM) for 20 min. at which time PG $F_{2\alpha}$ was added. LH (100 ng/ml) was added directly thereafter and incubation proceeded for 90 min. Each point represents the mean ± SEM of four replicates. A. Ouabain. B. Monensin.

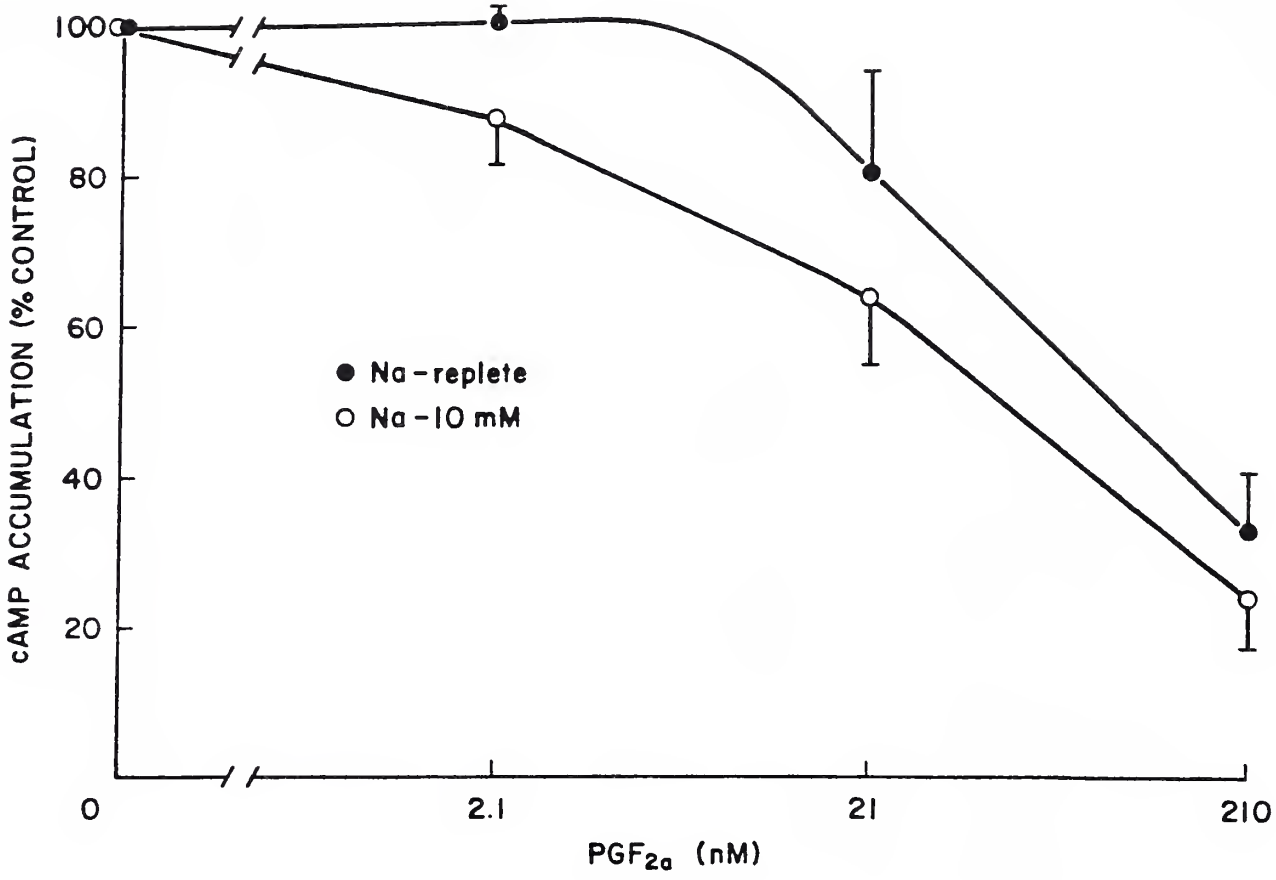

FIGURE 16. Effect of sodium depletion on inhibition by PG $F_{2\alpha}$ of LH-stimulated cAMP accumulation in luteal cells. Cells were washed and resuspended in sodium-replete medium or in medium in which sodium was replaced isotonically by choline chloride as described in Methods. PG $F_{2\alpha}$ was added to cells (10^5), and LH (100 ng/ml) was added directly thereafter. Incubation proceeded for 90 min. Each point represents the mean $\pm$ SEM for four replicates.

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