The Inositol Phosphate/Diacylglycerol Pathway in MA-10 Leydig Tumor Cells

ACTIVATION BY ARGinine VAsoPRESSIN AND LACK OF EFFECT OF EPIDERMAL GROWTH FACTOR AND HUMAN CHORIOGONADOTROPIN*

(Received for publication, November 14, 1988)

Mario Ascolil, Omar P. Pignataro, and Deborah L. Segaloff
From The Population Council, New York, New York 10021

It is now well established that mouse epidermal growth factor (mEGF) modulates the hormonal responsiveness of MA-10 Leydig tumor cells but does not affect cell multiplication. The studies presented herein are the first in a series of experiments designed to characterize the intracellular signaling systems activated by mEGF and their possible roles in mediating the diverse biological actions of this growth factor in MA-10 cells.

We show that (i) MA-10 cells express a hormone-sensitive inositol phosphate/diacylglycerol pathway that can be stimulated with arginine vasopressin (AVP), (ii) mEGF does not activate this pathway, and (iii) activation of this pathway with arginine vasopressin does not mimic the biological actions of mEGF. Other data presented show that lutropin/choriogonadotropin, the principal endocrine regulators of Leydig cell function, also do not stimulate the inositol phosphate/diacylglycerol pathway in MA-10 cells.

Using a clonal strain of cultured Leydig tumor cells (MA-10), we have previously shown that mouse EGF activates steroid biosynthesis without increasing cAMP accumulation (1) and modulates the actions of LH/CG at three different levels: the LH/CG receptor (2), the activation of adenylate cyclase (3), and step(s) localized beyond the formation of cAMP (1). Within minutes of addition of mEGF one can detect an attenuation of the ability of human CG to activate adenylate cyclase and steroid synthesis (1, 3). This inhibition, however, is only transient, occurring within the first hour of addition of mEGF. Thereafter, beginning at about 1 h and continuing to about 8 h, the inhibitory effect on the increased rate of cAMP accumulation induced by hCG disappears and mEGF becomes a potentiator of the activation of steroidogenesis by hCG and cAMP analogues (1). This potentiation is synergistic (rather than additive) and is detectable only with submaximal concentrations of hCG, cAMP analogues, or other compounds that increase cAMP accumulation (1). The third action of mEGF, a reduction in the number of LH/CG receptors has a much slower onset. Upon addition of mEGF there is a 6-8 h lag when LH/CG receptors do not change. At subsequent times the number of LH/CG receptors begins to decline and reaches a new steady state (corresponding to 15-30% of control) about 48 h after addition of mEGF (2). This reduction in LH/CG receptors is accompanied by a reduction in the ability of LH/CG to activate steroid synthesis. The steroidogenic response to cAMP analogues, however, remains unchanged (2).

An important step toward understanding these actions of mEGF would be to establish which intracellular signaling system(s) are activated by mEGF in MA-10 cells and which of these signaling systems are responsible for the actions of mEGF summarized above. Thus, we have initiated a series of experiments designed to answer these questions. While pursuing these studies we also thought that it would be appropriate to investigate the possibility that, in addition to activating adenylate cyclase, LH/CG might also activate the intracellular signaling system(s) that are activated by mEGF. This is an important question because although it is generally accepted that cAMP is an important mediator of the activation of steroidogenesis by LH/CG (4), it is not known with certainty if it is the only mediator of the actions of LH/CG on this pathway. This possibility is underscored by recent findings showing that steroid biosynthesis in normal Leydig cells (5, 6) or MA-10 cells (1) can be activated by "cAMP-independent pathways" and that cAMP is not the only second messenger system activated by LH/CG in Leydig (7) or granulosa/luteal cells (8-10).

We began these experiments by investigating the inositol phosphate/diacylglycerol pathway because this signaling system (i) is nearly as ubiquitous as the cAMP signaling system (see Refs. 11 and 12 for recent reviews); (ii) is involved in the hormonal control of steroid synthesis in other steroidogenic cells such as adrenal glomerulosa cells (13, 14); and (iii) is activated by mEGF in some (15-19), but apparently not all (20, 21), cell types. The results presented herein show that neither mEGF nor hCG activate the inositol phosphate/diacylglycerol pathway in MA-10 cells. We also show that AVP does activate this pathway in MA-10 cells, and it does not mimic the actions of mEGF or hCG.

EXPERIMENTAL PROCEDURES

Cells—The origin and handling of the MA-10 cells have been described (22). Cells were plated (in 6-well plates) on day 0 at a density of 3 x 10^5 cells/well in a total volume of 3 ml of growth medium (Waymouth MB752/1 modified to contain 1.1 g/liter NaHCO3, 20 mM Hepes, and 15% horse serum, pH 7.4). The wells were maintained in a humidified atmosphere containing 5% CO2 and

* This work was supported by Grant CA-40629 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: The Population Council, 1230 York Ave., New York, NY 10021. Tel.: 212-570-8766.

The abbreviations and trivial names used are: EGF, epidermal growth factor; CG, choriogonadotropin; LH, lutropin; AVP, arginine vasopressin; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; InsP3, inositol monophosphate; InsP4, inositol bisphosphate; InsP5, inositol triphosphate; PtdIns, phosphatidylinositol; PtdInsP2, phosphatidylinositol monophosphate; PtdInsP3, phosphatidylinositol bisphosphate.
were used on day 3. At this time, the cell density was 1.5–2.0 x 10^8 cells/well.

**Measurement of Inositol Phosphates and Phosphatidylinositols—**

Two days after plating the medium in the wells was changed to 2 ml of assay medium A (Waymouth MB752/1, modified to contain 1.1 g/liter NaHCO_3, 20 mM Hepes, and 1 mg/ml albumin buffer) containing 4–5 μCi/ml [^3H]inositol. The cells were incubated in this medium for 15–24 h prior to the experiment. At the end of the labeling period, the cells were washed five times with 2-ml aliquots of warm assay medium B (Waymouth MB752/1 without added NaHCO_3) containing 1 pM arachidonic acid. The removed washes were collected and the cell pellet was then scraped with 0.5 ml of cold 0.5 M HClO_4. The cells were then scraped with a rubber policeman and transferred to tubes. The cells were washed with 0.7 ml of cold HClO_4, and this wash was combined with the previous extract. After a 30-min incubation on ice, the extracts were centrifuged, and the supernatants and pellets were saved for analysis of the inositol phosphates and phosphatidylinositols, respectively.

The HClO_4 supernatants were neutralized by the addition of 0.6 ml of 0.72 M KOH/0.6 M KHC_O_3. The precipitated KClO_4 was removed by centrifugation, and the supernatant was mixed with 1 ml of assay medium A containing 1.1 g/liter NaHCO_3 and 20 mM Hepes, and 1 mg/ml albumin buffer to 10 ml. The supernatants were chromatographed on 0.5 x 3.0-cm columns of Dowex (formate form) as described by Berridge and coworkers (24). The columns were prewashed with 15 ml of 10 mM inositol. After sample application the columns were sequentially washed with 10 ml of 10 mM inositol, to wash residual [^3H]inositol, 5 ml of 5 mM sodium borate, 60 mM sodium formate (to elute glycerophosphoinositols), 0.1 M formic acid, 0.2 M ammonium formate (to elute InsP_3), 0.1 M formic acid, 0.4 M ammonium formate (to elute InsP_2), and 1.0 M ammonium formate (to elute InsP). In some experiments we did not attempt to separate the three inositol phosphates. In these cases, the filtrate containing the 0.2 M ammonium formate were eluted, and the total inositol phosphates were eluted with 10 ml of the 1.0 M ammonium formate solution. One-ml aliquots of each wash were mixed with 10 ml of BudgetSolve and counted in a liquid scintillation counter.

The phosphatidylinositols were measured as glycerophosphoinositol derivatives after alkaline hydrolysis of the lipid extracts (25). Lipids were extracted from the HClO_4 insoluble material (see above) with 4 ml of CHCl_3/CH_3OH/HCO_3 (100:100:1), and the phases were separated (by centrifugation) after addition of 0.75 ml of 10 mM EDTA. The lower phase was saved as the lipid phase. The upper phase was washed with 2 ml of CHCl_3. The lower phase from this mixture was then combined with the original lipid phase, and the resulting solution was washed with 1 ml of a 1:1 mixture of CHCl_3/HCO_3 (100:1) and 10 mM EDTA. The lower phase was then washed and dried under N_2. Each dried sample received 0.2 ml of CHCl_3, 0.3 ml of CH_3OH, 0.1 ml of 0.5 M NaOH, and 0.1 ml of 0.84 M formic acid. The mixture was then neutralized by the addition of 150 pl of 1 M LiCl. After adding 2 ml of warm assay medium B (with or without 20 mM LiCl), the wells were floated on a 37 °C water bath and preincubated for 15 min. The indicated hormones were added at the end of this preincubation. After incubation with the hormones for the indicated times, the wells were placed on ice, and the medium was quickly aspirated and replaced with 0.5 ml of cold 0.5 M HClO_4. The cells were then scraped into a 10 ml beaker and preincubated for 15 min. The indicated hormones were added at the end of this preincubation. After incubation with the hormones for the indicated times, the wells were placed on ice, and the medium was quickly aspirated and replaced with 0.5 ml of cold 0.5 M HClO_4. The cells were then scraped with a rubber policeman and transferred to tubes. The wells were washed with 0.7 ml of cold HClO_4, and this wash was combined with the previous extract. After a 30-min incubation on ice, the extracts were centrifuged, and the supernatants and pellets were saved for analysis of the inositol phosphates and phosphatidylinositols, respectively.

**RESULTS**

**AVP Stimulates the Accumulation of Inositol Phosphates in MA-10 Cells—**

After adding 1 ml of assay medium B containing 1 μM arachidonic acid, the wells were floated on a 37 °C water bath and preincubated for 5 min. The indicated hormones were added at the end of this preincubation. At the times indicated the wells were placed on ice, the medium was quickly aspirated, and the cells were layered with 1 ml of cold CHCl_3 containing about 10,000 cpm of [^3H]cholesterol (used to determine procedural losses, see below). The cells were then scraped with a rubber policeman and transferred to tubes. The wells were scraped a second time into 1 ml of the same solution, and this extract was combined with the previous one. The methanol extract was thoroughly mixed with 4 ml of CHCl_3. The phase was separated (by centrifugation) after the addition of 1 ml of H_2O, and the lower phase was collected and dried under N_2. The dried extract was redissolved in a minimal amount of CHCl_3/CH_3OH (2:1) and applied to Silica Gel thin layer plates. Neutral lipids were resolved by developing the plates twice in hexane/diethylether/acetic acid (75:25:2). The plates were stained with iodine vapor and the position of putative lipid classes was compared with that of authentic standards. This system gives a good separation of cholesterol esters (R_2 = 0.84), triacylglycerols (R_2 = 0.73), free fatty acids (R_2 = 0.47), and diacylglycerol (R_2 = 0.23). Monacetylglucers migrate only minimally (R_2 = 0.02) and the phospholipids remain at the origin. The different areas of the plates were then marked, cut, placed in scintillation vials containing 5 ml of BudgetSolve, and counted in a liquid scintillation counter. The data were corrected for the recovery of the [^3H]cholesterol oleate standard.

**Diacylglycerol Formation—**

Cells-Before starting any studies on the possible effects of mEGF and hCG on the formation of inositol phosphates and diacylglycerol, it was important to find a positive control that could be used to test the methodology used, and to verify the existence of this pathway in MA-10 cells. Thus, we tested a number of ligands that have been shown to increase the formation of inositol phosphates in MA-10 cells. For these experiments we tested mEGF and hCG, and several other agonists (muscarinic cholinergic agonists, and a2-adrenergic agonists) and found two, bombesin and AVP, that increased the formation of inositol phosphates in MA-10 cells. Of these two, we decided to further characterize the actions of AVP primarily because of its potential physiological significance. In this respect, previous studies from Hsueh and coworkers (31) have shown that normal rat Leydig cells bind AVP and that AVP is produced in the testis (reviewed in Ref 31).

Time courses of the effects of AVP on the accumulation of inositol phosphates and the levels of phosphatidylinositols in
**Inositol Phosphates in Leydig Tumor Cells**

**Fig. 1. Effects of AVP on the levels of inositol phosphates (A) and phosphatidylinositols (B) in MA-10 cells incubated without LiCl.** Cells were labeled with [3H]inositol, washed and preincubated in assay medium B without LiCl as described under "Experimental Procedures." AVP (1 μM) was added at the end of the preincubation (time = 0 in the figures) and the incubation was continued. The different inositol phosphates (A) and phosphatidylinositols (B) were quantitated as described under "Experimental Procedures." The results of a representative experiment are shown. Each point represents the mean (± S.E.) of triplicate wells.

MA-10 cells are shown in Figs. 1 and 2. In these experiments cells were prelabeled with [3H]inositol, and the levels of precursors and products of the inositol phosphate pathway were examined at different times after addition of 1 μM AVP.

**Fig. 2. Effects of AVP on the levels of inositol phosphates (A) and phosphatidylinositols (B) in MA-10 cells incubated with LiCl.** The experimental design was as described in the legend to Fig. 1, except that the assay medium used during the preincubation and incubation contained 20 mM LiCl. Additional details are provided under "Experimental Procedures." The results of a representative experiment are shown. Each point represents the mean (± S.E.) of triplicate wells.

One set of experiments (Fig. 1) was done in the absence of LiCl (an inhibitor of the degradation of some of the inositol phosphates, see Ref. 32 for a recent review) and the longest time point examined was 5 min. The other set (Fig. 2) was done in the presence of LiCl and the time course examined was extended to 30 min. The results presented in Fig. 1A show that AVP elicits a rapid increase in the appearance of InsP (1.5-fold), InsP₂ (5-fold), and InsP₃ (7-fold). The elevated levels of each of these products was already maximal at the earliest time point examined (15 s after addition of AVP), remained relatively constant for 1–2 min and showed a declining trend by 5 min. The results presented in Fig. 1B show that during this time period AVP did not induce a change in the levels of PtdIns. It induced a small but prolonged (also...
see Fig. 2B) decrease in the levels of PtdInsP, and a small and transient decrease in the levels of PtdInsP,2. It is important to note that the magnitude of the decrease in PtdInsP,2 is similar to the magnitude of the increase in InsP, thus suggesting a precursor-product relationship. The magnitude of the decrease in PtdInsP, however, is about 10-fold larger than the increase in InsP,2. The magnitude of this decrease is also about 10-fold larger than the amount of PtdInsP that would be needed to be converted to PtdInsP,2 to replenish and then maintain the amount of this compound at a steady state (center and lower panels, Fig. 1B).

The results presented in Fig. 2A show that in the presence of LiCl the inositol phosphates continue to accumulate for at least 30 min. InsP, accumulates in a linear fashion for the entire 30-min time period, while InsP,2 and InsP, are also observed in the cells incubated without LiCl (compare Figs. 1B and 2B). We do not know if the increase in PtdIns, PtdIns, PtdIns,3, and PtdIns,4, is similar to the magnitude of the increase in InsPs thus suggesting a precursor-product relationship. The magnitude of the increase in InsPs thus presented in Fig. 2B show that the changes described above were accompanied by an increase in the level of PtdIns, PtdIns,2, and a decrease in the level of PtdIns,2. The decrease in the level of PtdIns,2 was also observed in the cells incubated with LiCl, but the increase in the other two compounds was not observed in the cells incubated without LiCl (compare Figs. 1B and 2B).

MA-10 Cells Express Only the V1 Type of AVP Receptors—There are two types of AVP receptors, the V1 type receptors which are coupled to the inositol phosphate/diacylglycerol pathway, and the V2 type which are coupled to adenylyl cyclase. The V2 receptors have been identified in kidney, while the V1 receptors are expressed in a number of cell types such as hepatocytes and smooth muscle cells (see Ref. 33). Hsueh and co-workers (34) have previously shown that normal rat Leydig cells express V1 receptors. Additional evidence to support this conclusion is presented below.

Fig. 3. Effects of increasing concentrations of AVP and a V2 selective antagonist on the levels of inositol phosphates in MA-10 cells. Cells were labeled with [3H]inositol, washed and preincubated in assay medium B with 20 mM LiCl as described under "Experimental Procedures." The indicated concentrations of AVP were then added, and the incubations were continued for 30 min. Total inositol phosphates were measured as described under "Experimental Procedures." The results of a representative experiment are shown. Each point represents the mean (±S.E.) of triplicate wells. InsP,2 = total inositol phosphates.

TABLE I

| Additions                        | Total inositol phosphates (cpm × 10^-3)/10^6 cells |
|---------------------------------|-------------------------------------------------|
| None                            | 3,729 ± 191 (1.0)                               |
| AVP (0.1 μM)                    | 44,429 ± 2,903 (11.9)                           |
| Antagonist (10 μM)              | 4,326 ± 267 (1.2)                               |
| AVP (0.1 μM) + Antagonist (10 μM) | 5,058 ± 416 (1.4)                           |

Fig. 4. Effects of AVP and a V2 selective agonist on cAMP levels in MA-10 cells. Cells were incubated in 2 ml of assay medium A containing buffer only, AVP, dDAVP, or hCG as indicated. Incubations were done for 30 min or 4 h in the presence or absence of 0.5 mM 3-isobutyl-1-methylxanthine (MIX) as indicated. Intracellular cAMP was measured as described under "Experimental Procedures." The results of a representative experiment are shown. Each bar shows the mean (±S.E.) of triplicate wells. Note the different scales in the two panels.

studies did not include an analysis of the signaling system activated by AVP, they measured the binding affinities of several AVP analogues and concluded that normal rat Leydig cells express the V1 receptor (34). The data presented so far also suggest that, like their normal counterparts, MA-10 cells express the V1 receptor. Additional evidence to support this conclusion is presented below.

The results presented in Fig. 3 show the effects of increasing concentrations of AVP and deamino-[Arg5,vasopressin, a V2 selective agonist (35), on the production of inositol phosphates. With AVP a measurable effect was easily detected at 1 nM, and the half-maximal and maximal effects were detected at 10-20 and 100-1000 nM, respectively. These concentrations are somewhat higher than the concentrations of AVP that have been previously shown to stimulate this pathway in other cell types (36-39). As expected for a V1-mediated response, however, the V2 selective agonist was less effective than AVP in stimulating the formation of inositol phosphates. The results presented in Table I show that addition of a high concentration of a V1 selective antagonist (35) had little or no effect on the production of inositol phosphates, but it blocked the effects of AVP on the production of inositol phosphates almost completely. Last, the results presented in Fig. 4(left panel) show that AVP or the V2 selective agonists were unable to increase cAMP accumulation in MA-10 cells even in the presence of a phosphodiesterase inhibitor, while under the same experimental conditions (Fig. 4, right panel)
hCG, induced a marked increase in cAMP accumulation.

Taken together these results show that MA-10 cells express only the V1 type of AVP receptors. Thus, AVP stimulates the formation of inositol phosphates but does not increase cAMP levels in MA-10 cells.

**EGF and hCG Do Not Stimulate the Formation of Inositol Phosphates in MA-10 Cells**—The results summarized above show that MA-10 cells express a hormonally responsive phospholipase C and document the validity of the methodology used to measure the production of inositol phosphates. Having satisfied these criteria, we next determined the effects of mEGF and hCG on the levels of inositol phosphates and phosphatidylinositols. These experiments were done using a concentration of mEGF that elicited a maximal response in the cells. It is worth noting that we never observed increased concentrations of hCG used also elicits a maximal response with respect to cAMP accumulation (3, 4). The results presented in Table III show that both mEGF and hCG increased rather small (1.3-fold) increases in the levels of InsP in this experiment, but had no effect on the levels of InsP$_2$ and InsP$_3$. It is important to note that the magnitude and time course of the effect of mEGF and hCG on the accumulation of InsP (Fig. 5A) are very different from those observed with AVP (Fig. 2A), and that unlike the effects of AVP (Fig. 2A), mEGF and hCG do not increase the levels of InsP$_2$ and InsP$_3$ (Fig. 5A). Likewise, the effects of either of these two ligands on the levels of the different phosphatidylinositols (Fig. 5B) do not resemble those observed with AVP (Fig. 2B).

The results of several experiments are summarized in Table II and show that neither mEGF nor hCG have a significant effect on the accumulation of InsP, InsP$_2$, or InsP$_3$ in MA-10 cells. It is worth noting that we never observed increased accumulations of InsP$_2$ and InsP$_3$ in any of these experiments. A modest increase in the accumulation of InsP, however, was observed in some (see Fig. 5A for an example), but not in all, experiments. We are not sure about the significance (if any) of this finding. We are currently investigating the possibility that mEGF and hCG induce an increase in other inositol-containing compound(s) that are water soluble and elute from the Dowex columns in the same position as inositol phosphate. Our ability to detect these compound(s) would be variable if they were released into the medium rather than remaining in the cells, because the medium was aspirated prior to extracting the cells for the measurement of the inositol phosphates (see "Experimental Procedures" for details).

Taken together, our data show that mEGF and hCG do not stimulate the formation of inositol phosphates in MA-10 cells. These data are at variance with the results of other investigators who have reported that LH or hCG increase the formation of inositol phosphates in rat and porcine granulosa cells and bovine luteal cells (8–10).

**Effects of AVP, mEGF, and hCG on Diacylglycerol Production**—According to the classical pathway described in many other tissues, the increased formation of inositol phosphates should be accompanied by increased accumulation of diacylglycerol (reviewed in Ref. 11). Diacylglycerol, however, can also arise from the hydrolysis of other lipids (41, 42), and thus may be formed without increased formation of inositol phosphates. Therefore, it was important to determine if AVP, mEGF, or hCG increase diacylglycerol content in the MA-10 cells.

The results presented in Fig. 6 show that, as expected, AVP increased the levels of diacylglycerol in MA-10 cells. This effect was detectable at 2 min (the earliest time point tested). It reached a maximum at 4 min (1.4-fold increase) and remained elevated until at least 8 min. At longer times (20 and 30 min, data not shown) these levels returned toward basal. The results presented in Table III show that, in contrast to AVP, mEGF and hCG do not increase the levels of diacylglycerol in MA-10 cells.

**AVP Does Not Mimic the Actions of mEGF on MA-10 Cells**—Having established that MA-10 cells have the V1 type AVP receptors, and that AVP elicits the effects expected of a ligand that activates phospholipase C, we set out to determine
if AVP mimicked any of the actions of mEGF on MA-10 cells. We have previously documented four actions of mEGF on MA-10 cells. It (i) transiently attenuates the hCG-provoked increase in cAMP synthesis (1, 3); (ii) increases progesterone synthesis without increasing cAMP levels (1); (iii) potentiates the activation of steroidogenesis by submaximal concentrations of hCG or cAMP analogues (1); and (iv) down-regulates the LH/Cg receptors (2). The results presented in Fig. 7 show that AVP does not mimic the biological effects of mEGF. It does not down-regulate the LH/Cg receptor (Fig. 7, top panel), does not attenuate the initial increase in cAMP synthesis provoked by hCG (Fig. 7, center panel), and it does not activate progesterone synthesis (Fig. 7, bottom panel).

**DISCUSSION**

The experiments presented herein are the first in a series of experiment designed to characterize the intracellular signaling systems activated by mEGF and hCG in MA-10 Leydig tumor cells and to elucidate their possible roles in mediating the biological actions of these two hormones. Using AVP as a positive control, we show that: (i) MA-10 cells have a hormone-sensitive phospholipase C that can be stimulated with a polypeptide hormone (i.e., AVP), (ii) mEGF and hCG do not activate phospholipase C in MA-10 cells, and (iii) activation of this pathway with AVP does not mimic the biological actions of mEGF or hCG previously documented in this cell line. These data provide novel information about the actions of mEGF, hCG, and AVP, and the intracellular signaling systems of MA-10 cells.

The ability of mEGF to activate phospholipase C has received a great deal of attention in the past few years. It now
appears that mEGF is capable of activating this enzyme in some (15-19), but not all (20, 21), cell types. The reason(s) for this difference is not clearly understood, but the results presented herein (Fig. 5 and Tables II and III) show that MA-10 cells fall in the latter category. In a recent publication Olashaw and Pledger (21) showed that NIH 3T3 fibroblasts (which normally do not respond to mEGF with increased formation of inositol phosphates) can be "induced" to respond by raising their endogenous cAMP levels with cholera toxin and a phosphodiesterase inhibitor prior to adding mEGF. The same manipulation, however, failed to induce a mEGF response in MA-10 cells (data not shown).

The effects of LH/CG on the activity of phospholipase C have also received some attention recently. Thus, LH has been shown to stimulate the formation of inositol phosphates in rat (9) and porcine granulosa cells (10), as well as in bovine luteal cells (8). It does not appear to stimulate this pathway in rat luteal cells, however (43). We are not aware of any previous data on the effects of LH/CG on this pathway in Leydig cells. Thus, the data presented herein (Fig. 5 and Tables II and III) represent the first demonstration that hCG does not activate the inositol phosphate/diacylglycerol pathway in Leydig cells, and the second example of a cell type where the binding of hCG does not activate this pathway. Inasmuch as previous work has shown that other effects of gonadotropins on the formation of inositol phosphates and diacylglycerol in gonadal cells have been done with LH (another hormone that binds to the LH/CG receptor) rather than hCG, we decided to compare the effects of ovine LH and hCG on this pathway. Our results (not presented) showed that ovine LH was also unable to increase the levels of inositol phosphates or diacylglycerol in MA-10 cells. The reasons for the difference in the ability of LH/CG to increase inositol phosphate formation in different target cells is not presently understood. It is clear from the results obtained with AVP, however, that the lack of effect of mEGF or hCG on the formation of inositol phosphates and diacylglycerol in MA-10 cells is not due to the experimental protocol used or to the absence of some component(s) of this pathway.

The results presented here show, for the first time, that MA-10 cells have a hormone-sensitive phospholipase C and that AVP is a potent stimulator of this pathway. There are two types of AVP receptors, the V1 subtype which is coupled to the inositol phosphate/diacylglycerol pathway, and the V2 subtype which is coupled to the cAMP pathway (33). The following findings show that MA-10 cells express only the V1 subtype receptors: (i) AVP increases the levels of inositol phosphates and diacylglycerol (Figs. 1-3, 6, and Table III), (ii) a V2 selective agonist is less active than AVP in increasing the levels of inositol phosphates (Fig. 3), (iii) a V1 selective antagonist effectively blocks the AVP-induced increase in inositol phosphates (Table I), and (iv) AVP or a V2 selective agonist do not increase the levels of cAMP (Fig. 4). These findings are consistent with previous observations made by Hsueh and co-workers (reviewed in Ref. 31) in normal rat Leydig cells. They had previously demonstrated the presence of AVP receptors in normal rat Leydig cells, and using competition binding experiments they concluded that these receptors were of the V1 subtype (34). The effects of AVP on the signaling systems of normal Leydig cells, however, were not investigated. The presence of functional V1 subtype AVP receptors in Leydig cells (Ref. 34 and this paper), together with the finding that AVP is produced in the testes (44, 45), are of interest because they raise the possibility that AVP plays a physiologically important role (yet to be identified) in the regulation of Leydig cell functions.

Since neither mEGF nor hCG activate phospholipase C in MA-10 cells, it can be safely concluded that the biological actions of mEGF and hCG on MA-10 cells are not mediated by this intracellular signaling system. The lack of participation of this signaling system in mediating the biological actions of mEGF or hCG is also supported by the finding that AVP does not mimic the actions of these two hormones on MA-10 cells. Thus, while hCG increases cAMP and steroid synthesis, AVP does not (Figs. 4 and 7). Likewise, AVP was unable to mimic the effects of mEGF on the levels of LH/CG receptors, hCG-induced cAMP synthesis, or steroid synthesis (Fig. 7).

The lack of effect of AVP on hCG-activated cAMP synthesis and on the levels of LH/CG receptors (Fig. 7) are somewhat surprising, because these effects can be induced by the addition of Ca++ ionophores or phorbol esters. Thus, Rebois and co-workers (46) have previously shown that a short preincubation of Leydig tumor cells with phorbol esters leads to a decrease in the ability of hCG to activate adenylate cyclase in isolated membranes. We have confirmed these findings by measuring the hCG-enhanced rate of cAMP accumulation in intact cells. We have also shown that a short preincubation of MA-10 cells with A23187 or ionomycin results in an attenuation of the hCG-enhanced rate of cAMP accumulation (47). Although we have not measured the effects of AVP on cytosolic Ca++ or protein kinase C (Ca++/phospholipid-dependent enzyme) activity in MA-10 cells, we expect both to be elevated. Perhaps the levels of cytosolic Ca++ and protein kinase C activity attained in response to AVP are not high enough (or are of too short a duration) to attenuate the activation of adenylate cyclase by hCG. It is also possible that the effects of phorbol esters on hCG-activated adenylate cyclase activity are not mediated by the activation of protein kinase C (see Ref. 38 for an example of this phenomenon). Although it has been reported that 1-oleyl-2-acetyl glycerol and 1,2-dioleyl glycerol also attenuate hCG-activated adenylate cyclase (46), we have been unable to reproduce this finding using 1,2-dioctanoyl glycerol. The same can be said about the phorbol ester-induced reduction of LH/CG receptors. This finding, which was initially reported by Rebois and co-workers (46), has been reproduced by us, but only with phorbol esters. 1,2-Dioctanoyl glycerol does not reduce the levels of LH/CG receptors in MA-10 cells. Thus, the lack of effect of AVP on LH/CG receptors and hCG-activated adenylate cyclase could be due to the lack of involvement of protein kinase C in regulating these phenomena.

Hsueh and co-workers (31, 49) have previously reported that AVP stimulates the synthesis of pregnenolone and progesterone, while also inhibiting the conversion of progesterone to androgens in primary cultures of rat interstitial cells. We cannot evaluate the effects of AVP on the conversion of progesterone to androgens in MA-10 cells because these cells have little or no 17α-hydroxylase activity, an enzyme necessary for this conversion to occur (22). Our data, however, do show that AVP has no effect on the synthesis of progesterone, the major steroid product of MA-10 cells (Fig. 7). As previously shown with other hormonal responses, this may be due to intrinsic differences in the two experimental systems (1, 6, 50). Last, it should also be noted that although AVP stimulates the formation of inositol phosphates in rat adrenal glomerulosa and fasciculata cells, and in Y-1 adrenal cortical cells, it stimulates steroid synthesis only in the glomerulosa cells (37-39). Thus, the lack of effect of AVP on progesterone synthesis in MA-10 cells is not entirely unexpected.

---

1. M. Hafez and M. Ascoli, unpublished observations.
2. D. L. Segloff and M. Ascoli, unpublished observations.
In summary, our data provide a clear answer to the questions we set out to investigate. Thus, we can safely conclude that (i) mEGF or hCG do not activate phospholipase C in MA-10 cells, and (ii) activation of this pathway (with AVP) does not mimic the actions of mEGF or hCG on MA-10 cells.

Acknowledgments—We thank Florence Kaczorowski for assistance with the cell culture and Dr. Mohamed Hafez for performing the experiments summarized in the center panel of Fig. 7. We also thank Dr. Harry Haigler (University of California at Irvine) for his generous gift of mEGF.

REFERENCES

1. Ascoli M., Euffa J., and Segaloff, D. L. (1987) J. Biol. Chem. 262, 9196–9203
2. Ascoli M. (1981) J. Biol. Chem. 256, 179–183
3. Pereira, M. E., Segaloff, D. L., and Ascoli, M. (1988) J. Biol. Chem. 263, 9761–9766
4. Pereira, M. E., Segaloff, D. L., Ascoli, M., and Eckstein, F. (1987) J. Biol. Chem. 262, 6093–6100
5. Cooke, B. A., and Sullivan, M. H. F. (1985) Mol. Cell. Endocrinol. 41, 115–122
6. Verhoeven, G., and Cailleau, J. (1986) Mol. Cell. Endocrinol. 47, 99–106
7. Sullivan, M. H. F., and Cooke, B. A. (1986) Biochem. J. 236, 45–51
8. Davis, J. S., Weakland, L. L., Farese, R. V., and West, L. A. (1987) J. Biol. Chem. 262, 8515–8521
9. Davis, J. S., Weakland, L. L., West, L. A., and Farese, R. V. (1986) Biochem. J. 238, 597–604
10. Dimino, M. J., Snitzer, J., and Brown, K. M. (1987) Biol. Reprod. 37, 1125–1134
11. Berridge, M. J. (1987) Annu. Rev. Biochem. 56, 159–193
12. Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bros, T. E., Ishih, H., Bansal, V. S., and Wilson, D. B. (1986) Science 234, 1519–1526
13. Kojima, I., Kojima, K., Kreuter, D., and Rasmussen, H. (1984) J. Biol. Chem. 259, 14448–14457
14. Kojima, I., Kojima, K., and Rasmussen, H. (1985) J. Biol. Chem. 260, 9177–9184
15. Hepler, J. R., Nakahata, N., Lovenborg, T. W., DiGuiseppe, J., Herman, B., Earl, H. S., and Harden, T. K. (1987) J. Biol. Chem. 262, 2951–2956
16. Wahl, M., and Carpenter, G. (1988) J. Biol. Chem. 263, 7581–7590
17. Johnson, R. M., and Garrison, J. C. (1987) J. Biol. Chem. 262, 17285–17293
18. Pike, L. J., and Eakins, A. T. (1987) J. Biol. Chem. 262, 1644–1651
19. Wahl, M. I., Daniel, T. O., and Carpenter, G. (1988) Science 241, 968–970
20. Besterman, J. M., Watson, S. P., and Custreecassas, P. (1986) J. Biol. Chem. 261, 725–727
21. Olahay, N. E., and Pledger, W. J. (1988) J. Biol. Chem. 263, 1111–1114
22. Ascoli, M. (1981) Endocrinology 108, 88–95
23. Martin, T. F. J. (1983) J. Biol. Chem. 258, 14816–14822
24. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P., and Irvine, R. F. (1983) Biochem. J. 212, 473–482
25. Kates, M. (1986) Techniques of Lipidology: Isolation, Analysis and Identification of Lipids, pp. 396–399. Elsevier Scientific Publishing Co., Amsterdam.
26. Monaco, M. E. (1987) Methods Enzymol. 141, 83–91
27. Ascoli, M., and Puett, D. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 99–102
28. Buettner, K., and Ascoli, M. (1984) J. Biol. Chem. 259, 15078–15084
29. Lloyd, C. E., and Ascoli, M. (1983) J. Biol. Chem. 96, 521–526
30. Segaloff, D. L., and Ascoli, M. (1981) J. Biol. Chem. 256, 11420–11423
31. Kasson, B. G., Adashi, E. Y., and Hsueh, A. J. W. (1986) Endocrine Rev. 7, 156–168
32. Majerus, P. W., Connolly, T. M., Bansal, V. S., Inhorn, R. C., Ross, T. S., and Lips, D. L. (1986) J. Biol. Chem. 263, 3051–3054
33. Jard, S. (1983) Curr. Topics Membr. Transp. 18, 225–285
34. Meidan, R., and Hseuh, A. J. W. (1985) Endocrinology 116, 416–423
35. Sawyer, W. H., Grzonka, Z., and Manning, M. (1981) Mol. Cell. Endocrinol. 22, 117–134
36. Bone, E. A., Fretten, P., Palmer, S., Kirk, C. J., and Michell, R. H. (1984) Biochem. J. 221, 803–811
37. Gallo-Fayet, N., Guillorn, G., Noelle Baillere, M., and Jard, S. (1986) Endocrinology 119, 1042–1047
38. Woodcock, E. A., McLeod, J. K., and Johnston, C. I. (1986) Endocrinology 118, 2432–2436
39. Langelis, D., Arrou, M., Saz, J. M., and Begeot, M. (1988) Mol. Cell. Endocrinol. 58, 199–205
40. Ascoli, M. (1985) in The Receptors (Conn, P. M., ed) Vol. 2, pp. 368–400, Academic Press, New York
41. Besterman, J. M., Duronio, V., and Custreecassas, P. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6785–6789
42. Sattil, A. R., Fox, J. A., Sherline, P., and Custreecassas, P. (1986) Science 233, 967–972
43. Labah, M., West, L. A., and Davis, J. S. (1988) Endocrinology 123, 1044–1052
44. Kasson, B. G., and Hseuh, A. J. W. (1986) Endocrinology 118, 25–31
45. Kasson, B. G., Meidan, R., and Hseuh, A. J. W. (1985) J. Biol. Chem. 260, 5302–5307
46. Robois, R. V., and Patel, J. (1985) J. Biol. Chem. 260, 8026–8031
47. Pereira, M. E., Segaloff, D. L., and Ascoli, M. (1988) Endocrinology 122, 2232–2239
48. Malvoisin, E., Wild, P., and Zwingelstein, G. (1987) FEBS Lett. 215, 175–178
49. Adashi, E. Y., and Hseuh, A. J. W. (1982) J. Biol. Chem. 257, 1301–1308
50. Welsh, T. H., Jr., and Hseuh, A. J. W. (1982) Endocrinology 110, 1499–1506