Human chorionic gonadotropin activates the inositol 1,4,5-trisphosphate-Ca^{2+} intracellular signalling system in bovine luteal cells

John S. Davis, Leigh A. West, Laura L. Weakland and Robert V. Farese

James A. Haley Veterans Administration Hospital and Department of Internal Medicine, University of South Florida College of Medicine, Tampa, FL 33612, USA

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Human chorionic gonadotropin, hCG, a hormone which increases intracellular cAMP, provoked rapid (30 s) and sustained (up to 30 min) increases in the levels of inositol mono-, bis- and trisphosphates (IP, IP_2 and IP_3, respectively) in bovine luteal cells. LiCl (10 mM) enhanced inositol phosphate accumulation in response to hCG. Concentration-dependent increases in inositol phosphates, cAMP and progesterone accumulation were observed in hCG-treated luteal cells. hCG also induced rapid and concentration-dependent increases in cytosolic free Ca^{2+} as measured by quin 2 fluorescence. These findings demonstrate that hCG stimulates the phospholipase C-IP_3 and diacylglycerol 'second messenger' system in the bovine corpus luteum.

Ca^{2+} Gonadotropin Inositol phospholipid Inositol phosphate (Corpus luteum)

1. INTRODUCTION

cAMP is recognized as a primary 'second messenger' of gonadotropin action in the corpus luteum [1]. Other studies have demonstrated that Ca^{2+} may also modulate the stimulatory action of LH and cAMP on progesterone synthesis [2–4], although it is not known whether gonadotropins alter intracellular Ca^{2+} levels. Recently, strong support has accumulated linking Ca^{2+} mobilization to increases in IP_3 produced by receptor-mediated hydrolysis of PIP_2 [5]. However, hormones which utilize cAMP as their intracellular second messenger are not thought to increase second messengers derived from inositol phospholipid hydrolysis [5–9]. In this study, we have examined the effect of hCG on inositol phospholipid metabolism and $[Ca^{2+}]_i$ as determined by quin 2 fluorescence. We report that hCG, in addition to increasing cAMP, provokes increases in IP_3 and $[Ca^{2+}]_i$ in isolated bovine luteal cells.

Abbreviations: hCG, human chorionic gonadotropin; $[Ca^{2+}]_i$, cytosolic free calcium concentration; quin 2/AM, quin 2 tetraacetoxymethyl ester; IP_3, inositol trisphosphate; IP_2, inositol bisphosphate; IP, inositol phosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-biphosphate; DAG, 1,2-diacylglycerol

2. MATERIALS AND METHODS

Corpora lutea were obtained from cows during early pregnancy, sliced, and dispersed with collagenase as described [10]. Luteal cell preparations ($1–1.5 \times 10^7$ cells/ml) were preincubated for 3 h in medium containing *myo*-[2-^{3}H]inositol (50 μCi/ml) under an atmosphere of 95% O_2, 5% CO_2 at 37°C. After this prelabeling period, the cells were washed and incubations were performed in triplicate with $0.5–1 \times 10^6$ viable cells, in a final volume of 0.5 ml of medium 199 containing 25 mM Hepes and 0.1% BSA under an atmosphere of 95% O_2/5% CO_2 at 37°C. After a

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15 min incubation with or without 10 mM LiCl control media or hormones were added and the incubations were continued for up to 60 min. Incubations were terminated by the addition of 0.5 ml ice-cold 10% trichloroacetic acid. [^{3}H]Inositol phosphates were purified as in [11,12] on columns (7 × 13 mm) of BioRad AG 1-X8 ion-exchange resin.

In some experiments, the acid-precipitable pellet was extracted with 5 ml of chloroform/methanol/HCl (200:100:0.75, by vol.) to determine the [^{3}H]inositol content of cellular inositol phospholipids. Phospholipids were purified by thin-layer chromatography as described in [13]. Progesterone and cAMP were measured by radioimmunoassay [1,10].

Cytosolic free Ca^{2+} concentrations, $[Ca^{2+}]_i$, were measured by the method of Tsien et al. [14] with minor modifications as described [11,15]. Luteal cells (10^7 cells/ml) were incubated with 100 μM quin 2/AM. The unhydrolyzed ester was removed and cellular fluorescence (3–5 × 10^6 cells/ml) signals derived from the quin 2-Ca^{2+} complex were measured at an excitation wavelength of 339 nm (slit 5 nm) and emission wavelength of 492 nm (slit 10 nm). All measurements were made using a Perkin-Elmer MPF-44A spectrofluorometer equipped with a magnetic stirrer and a thermostatted (at 37°C) cell holder. Maximum (F_{max}) and minimum (F_{min}) fluorescence were measured by rapidly saturating intracellular quin 2 with Ca^{2+} (F_{max}) by permeabilizing the cells with 0.2% Triton, and by adding 5 mM EGTA in Tris buffer, pH 8.5, to determine the basal fluorescence (F_{min}). No corrections were required for intrinsic fluorescence of the cells, media, hormones, and detergents. $[Ca^{2+}]_i$ was calculated according to the equation [11,14,15]:

$$[Ca^{2+}]_i = 115 \text{ nM } (F - F_{min})/(F_{max} - F).$$

3. RESULTS

hCG provoked rapid (30 s) increases in the levels of inositol polyphosphates (fig.1) with significant increases in IP levels occurring after 1–2 min of hCG treatment. The initial rate of IP_3 formation was greater than the rate of IP_2 formation. Further increases in the levels of all inositol phosphates

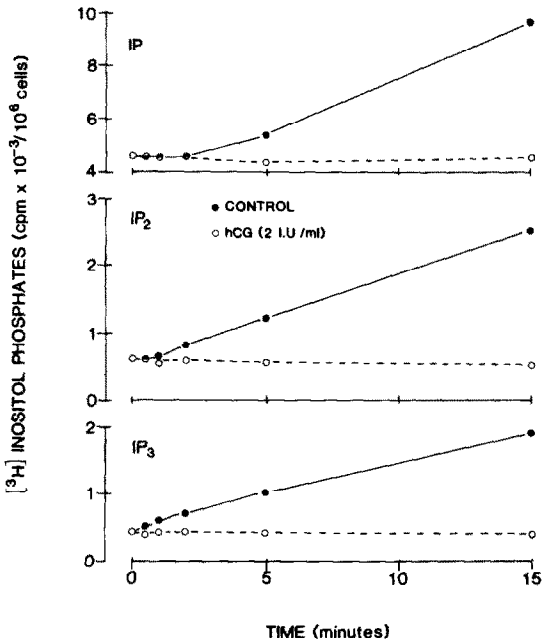

Fig.1. Temporal profile of [^{3}H]inositol phosphate accumulation in response to hCG. Bovine luteal cells were preincubated for 15 min with 10 mM LiCl and were further incubated for up to 15 min with 2 IU/ml of hCG. Results are means of triplicate determinations in a representative experiment. Individual values varied less than 5% from the mean. Similar temporal relationships were observed in two other experiments.

were observed throughout 60 min of incubation (not shown). In other experiments with 10 mM LiCl (to inhibit inositol phosphate phosphatase activity) a significant enhancement of hCG-induced accumulation of inositol monophosphate was observed in incubations lasting 15 min (table 1). In similar incubations, levels of [^{3}H]PI were increased 32% after 30 min treatment with hCG: 24834 ± 634 vs 32971 ± 1016 cpm/5 × 10^5 cells (mean ± SE, n = 3). Smaller increases (15–20%) were observed in labeling of PIP and PIP_2 (not shown). To evaluate further the response to hCG, studies were conducted to determine the lowest effective concentration that would produce an increase in inositol phosphate levels. hCG at 2–20 IU/ml in incubations with 10 mM LiCl provoked maximal increases in inositol phosphate accumulation (fig.2). A slight stimulatory response was observed at 0.002 IU/ml of hCG. As expected, hCG also

Table 1

Effect of LiCl on hCG-induced inositol phosphate accumulation in bovine luteal cells

[^{3}H]Inositol metabolite	Radioactivity in metabolite (cpm)			
	− LiCl		+ LiCl	
	Control	hCG	Control	hCG
IP	4108 ± 8	7146 ± 68[a]	4561 ± 85[a]	9565 ± 115[a,b,c]
IP_2	486 ± 18	2564 ± 51[a]	525 ± 34	2567 ± 63[a,b]
IP_3	399 ± 15	1727 ± 40[a]	391 ± 17	1898 ± 29[a,b]

[a] vs control (− LiCl), $p < 0.05$
[b] vs control (+ LiCl), $p < 0.05$
[c] vs hCG (− LiCl), $p < 0.05$

Bovine luteal cells were preincubated for 15 min with or without 10 mM LiCl and were further incubated for 15 min with 2 IU/ml of hCG. Results are expressed as mean ± SE, $n = 3$

provoked concentration-dependent increases in cAMP and progesterone in these incubations (fig.2). However, exogenous cAMP (5 mM 8-bromo-cAMP) and forskolin (10 μM) were unable to stimulate phosphoinositide metabolism (not shown).

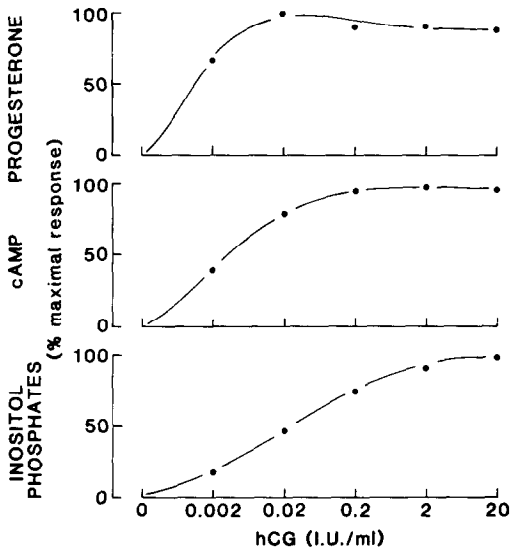

Fig.2. Dose dependency of hCG-induced increases in inositol phosphate, cAMP and progesterone accumulation. Bovine luteal cells were preincubated for 15 min with 10 mM LiCl and were further incubated for 30 min with 0.002–20 IU/ml of hCG. Results are means of 2 or 3 experiments after expression as a percentage of the maximal response observed in each experiment; the average variation was less than 10% of the mean.

Fig.3 shows the effect of hCG on $[Ca^{2+}]_i$ levels in quin 2-loaded bovine luteal cells. The increases in $[Ca^{2+}]_i$ observed in response to hCG were maximal in the first 30–60 s after hormone addition and were sustained at a slightly lower level for up to 10 min. As determined in 11 determinations from 3 separate cell preparations, hCG significantly increased $[Ca^{2+}]_i$ (control vs hCG, 96 ± 11 vs 152 ± 22 nM Ca^{2+}, mean ± SE, $n = 11$). hCG provoked similar increases in $[Ca^{2+}]_i$ when luteal cells were incubated in Ca^{2+}-free media (not shown). In

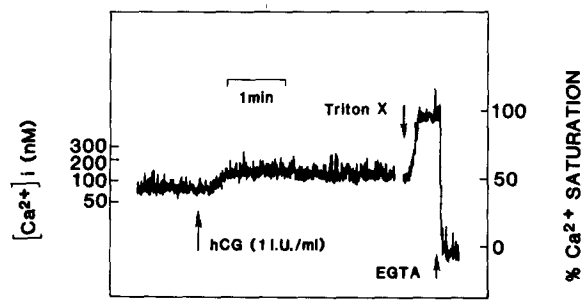

Fig.3. Effect of hCG on quin 2 fluorescence in isolated bovine luteal cells. The arrow indicates the addition of hCG (2 IU/ml). Maximum and minimum fluorescence were determined by addition of Triton-X and EGTA, respectively.

Ca^{2+}-free medium containing 1 mM EGTA basal $[Ca^{2+}]_i$ was reduced to 50–75 nM. Under these conditions hCG was able to increase $[Ca^{2+}]_i$, however, the response was reduced and was of shorter duration, lasting only 2–4 min. Similar to our results on IP_3 accumulation, hCG provoked concentration-dependent increases in $[Ca^{2+}]_i$. Maximal increases in $[Ca^{2+}]_i$ were observed at 2 IU/ml of hCG (the increases in $[Ca^{2+}]_i$ were 56 ± 11, 46 ± 14 and 24 ± 13 nM Ca^{2+} for 2, 0.2, and 0.02 IU/ml of hCG, respectively).

4. DISCUSSION

The results of these studies demonstrate that the action of hCG in bovine luteal cells is associated with increases in inositol phosphate accumulation. The initial rate of formation of IP_3 was greater than the rate of formation of IP_2 or IP suggesting that one of the earliest effects of hCG on cellular inositol phospholipid metabolism is the hydrolysis of PIP_2. It is unknown, however, whether IP_2 is formed directly from hCG-stimulated breakdown of PIP or if IP_2 is a product of IP_3 degradation. The more delayed increases in IP levels observed in these studies probably reflect the degradation of IP_3 and IP_2, as well as the formation of IP directly from PI hydrolysis. The sustained increases in the accumulation of inositol phosphates, together with the continued incorporation of [^{3}H]inositol into inositol phospholipids in the presence of hCG, suggest that hCG-induced inositol phospholipid synthesis and hydrolysis is a continuous process.

The present results also demonstrate that hCG increases $[Ca^{2+}]_i$ in bovine luteal cells. The possibility that IP_3 may be a second messenger for Ca^{2+} mobilization in bovine luteal cells is supported by the similar dose-response and temporal relationships between hCG-induced IP_3 formation and $[Ca^{2+}]_i$ mobilization. IP_3, through interaction with its intracellular receptor [16], produces a transient release of Ca^{2+} from an ATP-dependent, non-mitochondrial, vesicular pool, presumably the endoplasmic reticulum [5,17]. IP_3 also mimics calcium-dependent processes when microinjected into a variety of cells [5,18]. In the ovary, Ca^{2+} participates in gonadotropin-induced cAMP production [4], steroid synthesis [2–4] and secretion [3], ornithine decarboxylase induction [19], proteoglycan production [20] and Ca^{2+}-dependent protein phosphorylation [21–24]. These studies further implicate Ca^{2+} in the action of gonadotropins, but the exact role of increases in IP_3 and $[Ca^{2+}]_i$ in response to hCG remains to be determined.

Increased levels of DAG occur concomitantly with receptor-mediated phospholipase C-induced hydrolysis of inositol phospholipids [5–9,11]. Like cAMP and Ca^{2+}, DAG may play a central role in the action of hCG. DAG and the DAG-like tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate, TPA, activate protein kinase C [6,25]. The presence of protein kinase C in the bovine corpus luteum [24] and the increase in inositol phospholipid hydrolysis in hCG-stimulated luteal cells also point to the involvement of protein kinase C in the action of hCG. TPA stimulates progesterone production in bovine luteal [26] and rat granulosa [27] cells by a mechanism independent of increases in cAMP. On the other hand, it has also been shown that TPA inhibits gonadotropin-induced steroidogenesis in bovine luteal [28] and rat granulosa cells [29]. Other recent studies suggest that increases in DAG and activation of protein kinase C may be part of a negative feedback mechanism for the regulation of cellular activity [30,31].

In summary, we have demonstrated that hCG stimulates rapid and concentration-dependent increases in IP_3 and $[Ca^{2+}]_i$. These studies provide the initial evidence that hCG may provoke its cellular response by stimulating two distinct second messenger systems, (i) adenylate cyclase-cAMP and (ii) phospholipase C-IP_3 and DAG. In studies to be reported elsewhere, we have also observed increased levels of IP_3 in rat granulosa cells in response to luteinizing hormone [32] and hCG (unpublished).

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