Human chorionic gonadotropin and free beta subunits stimulate phospholipid methylation in intact rat Leydig cells

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The effect of human chorionic gonadotropin (hCG) on intact Leydig cell phospholipid methylation was studied. Hormonal stimulation of rat Leydig cells increased the incorporation of [methyl-^{3}H]methionine into phospholipids threefold. This effect was observed after 10 minutes of incubation time and was time and dose dependent with a maximal stimulation at 67 ng/ml of hCG. In the presence of hCG, ^{3}H-labeled methyl groups were preferentially incorporated into phosphatidyl-N-monomethylethanolamine. This effect of hCG was not reproduced by dibutyryl cyclic adenosine monophosphate (cAMP), cholera toxin, or forskolin. Purified hCGβ subunit but not hCGα subunit had stimulatory activity on Leydig cell phospholipid methylation. We conclude that luteinizing hormone (LH)/hCG stimulates specifically Leydig cell phospholipid methylation, because LH-releasing hormone or [Arg^8]-vasopressin did not modify these reactions. We postulate that these reactions are occurring at a cellular level that involves hormone-receptor interaction. It is also suggested that this biological response involves hCGβ subunit receptor interaction and does not require cAMP synthesis. (*Steroids* **58:** 314–319, 1993)

Keywords: steroids; Leydig cells; steroidogenesis; phospholipid methylation; human chorionic gonadotropin; luteinizing hormone

Introduction

It is now well documented that in rat Leydig cells, steroidogenesis is stimulated by luteinizing hormone (LH)/hCG through specific membrane receptors, which induces cyclic adenosine monophosphate (cAMP) production and finally an increased testosterone synthesis.[1]

It has been proposed that in several somatic cells, signal transduction mechanisms are related to plasma membrane phospholipid methylation.[2–8] Phospholipid methylation involves the enzymatic conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) via synthesis of phosphatidyl-N-monomethylethanolamine (PME) and phosphatidyl-N,N-dimethylethanolamine (PDE). S-Adenosylmethionine (SAM) constitutes the intracellular physiological methyl donor for the phospholipid methylation pathway. It has been demonstrated that different ligands enhance the incorporation of radioactivity from [*methyl*-^{3}H]methionine or [^{3}H]-SAM into phospholipid (PL) in a variety of cells.[2,4–8] However, there are conflicting results regarding phospholipid methyltransferase (PLMT) activity in vitro and the conversion of PE to PC in intact cells.[5–7] Possible phospholipid methylation functions are related to an increased binding capacity of hormones to their specific receptors,[8,9] a stimulation of the adenylate cyclase activity,[2] and an increased influx of ions into several somatic cells.[10,11]

In Leydig cells, high levels of PLMT activity, which can be stimulated by hCG and cAMP analogs, have been observed.[4] Furthermore, two known phospholipid methylation inhibitors (3-deazaadenosine and homocysteine thiolactone) were able to inhibit testosterone production and decrease the number of LH receptors.[3,8] In addition, PC may have a regulatory role on some of the enzymes associated with the steroidogenic pathway.[12] These observations suggest that phospholipid methylation could be involved in Leydig cell steroidogenesis. However, to date it has not been possible to establish a clear relationship between LH/hCG

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and increased phospholipid methylation in Leydig cells.[13]

In this work, we have designed experiments to study the effect of LH/hCG on phospholipid methylation in intact Leydig cells. Results demonstrate that LH/hCG exerts a time- and dose-dependent stimulation of [*methyl*-^{3}H]methionine incorporation into phospholipid of purified Leydig cells. A possible mechanism for these reactions, including a role for the free hCGβ subunit, is also discussed.

Experimental

Materials

hCGβ subunit (CR 125) and hCGα (CR mix) were prepared under the supervision of Dr. S. Birken, Center for Population Research of NICHD, National Institutes of Health. Hybrid hCG (ABIER-CR-1XY) was prepared by recombining hCGα and hCGβ subunits (Canfield's hCGα-CR 123 and hCGβ-CR 123). HCG (3,000 IU/mg), [Arg^8]-vasopressin (AVP), Percoll, Hepes, dibutyryl cAMP (dbcAMP), 3-isobutyl-1-methylxanthine (MIX), L-homocysteinethiolactone, L-α-phosphatidylcholine (PC), DL-α-lysophosphatidylcholine (LPC), L-α-phosphatidylethanolamine (PE), DL-α-phosphatidyl-N-monomethylethanolamine (PME), DL-α-phosphatidyl-N,N-dimethylethanolamine (PDE) were from Sigma Chemical Co., St. Louis, MO. LH-releasing hormone (LHRH) agonist (D-Ser (But)^{6}des-Gly^{10}-NH$_2$LHRH-ethylamide) was from Hoechst A.G., Frankfurt, Germany. Cholera toxin (19.7 Lb/μg protein, where Lb is the limit of blueing) and forskolin, were from Calbiochem-Behring Corp., CA, USA. L-[*methyl*-^{3}H]methionine (15 Ci/mmol) was from Amersham International, Bucks, England. Collagenase was from Worthington Biochemical Co., Freehold, NJ. 3-Deazaadenosine (3-DZA) was synthesized by Southern Research Institute, Birmingham, AL.

Animals

Adult male Wistar rats (60 days old) from our colony were used. Animals were maintained under controlled conditions of 12 hours light : 12 hours darkness, with rat chow and water available ad libitum. Animals were first anesthetized and killed by decapitation.

Cell preparation

Testes were removed and perfused via testicular artery with sodium phosphate buffer (PBS) to remove blood cells. Testicular cells were dispersed by collagenase, and Leydig cells were purified through a Percoll gradient as previously described.[14] Briefly, interstitial cells (1.5×10^7) were applied to a 26-ml linear 10% to 80% Percoll gradient in Hepes buffer, pH 7.4,[15] and centrifuged at $800 \times g$ for 30 minutes. Gradient fractions (1 ml) were collected and fractions 20 to 24 were pooled.[15] Leydig cell number was established by a histochemical reaction for 3β-hydroxysteroid dehydrogenase (3β-HSD).[16] Cell viabilities as assessed by trypan blue-exclusion, and the percentage of cells 3β-HSD positive in those fractions, were 95% and 85%, respectively.

Phospholipid methylation

Approximately 0.5×10^6 Leydig cells were preincubated in a supplemented Tyrode's buffer, pH 7.3 (containing 116.1 mM NaCl, 10 mM KCl, 0.3 mM Na_2HPO_4, 24.9 mM $NaHCO_3$, 100 mg% glucose, 0.125 mM piruvate, 6.25 mM lactate, and 0.1% bovine serum albumin), with 10 μCi of L-[*methyl*-^{3}H]methionine (21.3 μM) for 10 minutes. After this period, aliquots of buffer containing hCG, dbcAMP, cholera toxin, hCGα subunit, hCGβ subunit, hCG hybrid, or buffer (controls) were added and incubated for 30 minutes at 34C under an atmosphere of 95% O_2/5% CO_2. The reaction was stopped by the addition of 0.5 ml of a cold PBS–sucrose buffer pH 7.3 (buffer A) as previously described.[10] The cellular pellet obtained after centrifugation at $1000 \times g$, was immediately frozen in liquid N_2 and kept at −80C until phospholipids extraction. Phospholipids were extracted in 3 ml of a chloroform mixture containing chloroform/methanol/2N HCl (12 : 6 : 1; v/v/v) as previously described.[10] The final chloroform phase volume (1 ml) was dried at 60C under N_2 and analyzed by liquid scintillation spectrometry.

Separation and analysis of methylated phospholipids

The extracted phospholipids from control and hCG-treated Leydig cells were separated and identified by thin-layer chromatography as previously described.[10] Briefly, the dried chloroform phase was redissolved in 0.4 ml of chloroform/methanol mixture (2 : 1; v/v). Subsequently, 20 μg each of LPC, PC, PME, PDE, and PE were added to a 0.2-ml aliquot of the extracted Leydig cell phospholipids. Next, the sample containing the methylated phospholipids and the carriers was applied to a prescored silica gel G plate. Also, a mixture of carrier phospholipids and single phospholipids was applied to the same plate. The ascending chromatography was developed with a mixture of propionic acid/n-propyl alcohol/chloroform/water (2 : 2 : 1 : 1; v/v/v/v) for phospholipid separation.[12] After the solvent front had migrated approximately 14 cm, the plate was dried, standard phospholipids were visualized with iodine vapors, and their chromatographic mobilities determinated. The prescored section of the plate corresponding to the sample was divided into 5-mm bands. Each band was scraped and eluted with 0.6 ml of the chromatography solvent. After elution, tubes were centrifuged at $800 \times g$ for 10 minutes and 0.4 ml were dried and finally analyzed by liquid scintillation spectrometry.

Testosterone production

Aliquots of resuspended cells (0.5×10^6 cells/ml) were incubated in the absence or presence of hCG in medium M-199 for 3 hours at 34C with continuous shaking. Testosterone was determined by radioimmunoassay as previously described.[16] The interassay and intraassay coefficient of variation of standard samples were 13.5% and 8%, respectively.

General procedures

Radioactivity was measured in a Beckman LS 5000 TD liquid scintillation spectrometer. Data are expressed as means ± SD and differences between groups were evaluated using Student's t test. Differences were considered significant if $P < 0.05$.

Results

Dose-response of action of hCG on intact Leydig cell phospholipid methylation

Figure 1 shows that hCG significantly stimulated PL methylation in intact Leydig cells compared with control. This stimulatory effect was dose-dependent with an ED_{50} of approximately 5 ng/ml and maximal stimulation at 67 ng/ml. On the other hand, maximal testosterone production was obtained at 6.7 ng/ml of hCG with an ED_{50} of 1.2 ng/ml. Similar results were obtained

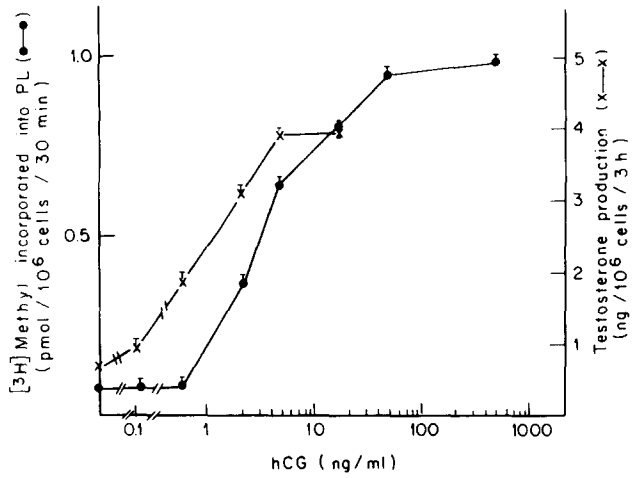

Figure 1 Effect of hCG concentration on phospholipid methylation and testosterone production. Phospholipid methylation and testosterone production were determined as described in Experimental. Data represent the mean ± SD of four experiments run in triplicate.

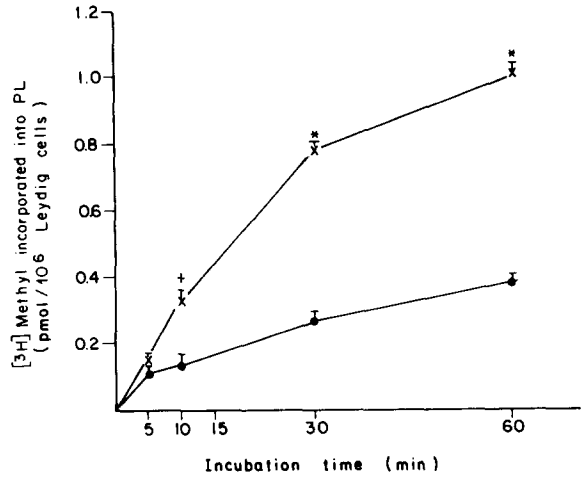

Figure 2 Time course of phospholipid methylation in rat Leydig cells. Approximately 0.5 × 10^6 Leydig cells were preincubated with 10 μCi of [*methyl*-^{3}H]methionine (21.3 μM). After 10 minutes, hCG (670 ng/ml, -x-) or buffer (control, -●-), was added and the incubation continued for variable times. Values are expressed as the mean ± SD of three experiments run in triplicate. $+P <$ 0.01; $*P < 0.005$ compared with the respective control (Student's *t* test).

when experiments were performed with rat LH in identical conditions (data not shown).[15]

Time course of action of hCG on intact Leydig cell phospholipid methylation

Incubation of purified Leydig cells with 670 ng/ml caused a time-dependent stimulation of [^{3}H]methyl group incorporation into PL (Figure 2). This effect was apparent 10 minutes after addition of the hormone. The maximal threefold activation of PL methylation was attained after 30 minutes of incubation in the presence of hCG. This stimulatory effect was maintained throughout the entire incubation period and was not due to an increased cell permeability to [*methyl*-^{3}H]methionine (data not shown). Indeed, [^{3}H]methyl detected in labeled acid-soluble compounds was not changed in control or hCG-treated cells.[10]

Separation and analysis of methylated PL

Quantitative separation of the methylated PL from control Leydig cells by thin-layer chromatography showed that [^{3}H]methyl groups were preferentially incorporated into PC and PDE; PME was labeled to a lesser extent (Figure 3). Although hCG-treated Leydig cells showed an increased incorporation of [^{3}H]methyl groups into all PL analyzed, the largest stimulatory effect observed was on PME (threefold).

Effect of LHRH and AVP on testosterone production and on phospholipid methylation of Leydig cells

Because direct effects of LHRH and AVP on gonadal steroidogenesis have been previously reported,[17,18] we analyzed whether these hormones were able to modify Leydig cell phospholipid methylation. As shown in Table 1, treatment of purified Leydig cells with 0.8 nM LHRH or 0.1 μM AVP for 30 minutes did not change [^{3}H]methyl groups incorporation into PL. In addition, either LHRH or AVP did not modify hCG-stimulated PL methylation. HCG-stimulated testosterone production decreased when LHRH (0.8 nM) or AVP (0.1 μM) was added to the incubation medium.

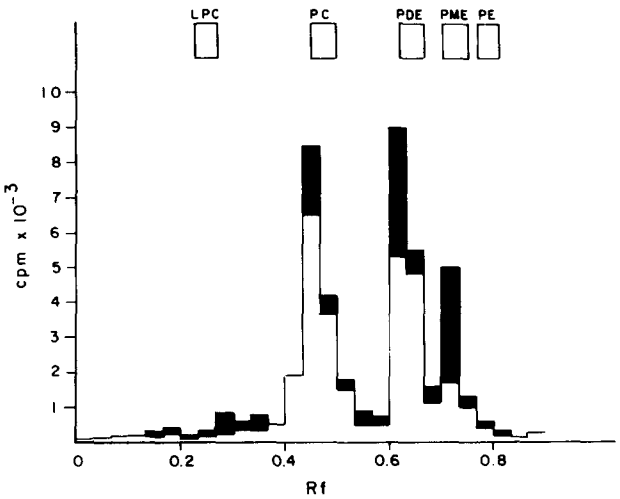

Figure 3 Separation of methylated phospholipids. **Open bars**: control cells. **Solid bars**: hCG-treated cells. PL extracted from control and hCG-incubated Leydig cells for 30 minutes were separated by thin-layer chromatography and quantitated. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidyl-N-monomethylethanolamine; PDE, phosphatidyl-N,N-dimethylethanolamine; Rf, chromatographic mobility. Eighty-five percent of the total organic soluble radioactivity was recovered. Results represent the methylated phospholipids extracted from 1 × 10^6 Leydig cells.

Table 1 Effect of LHRH and AVP on testosterone production and on intact Leydig cell phospholipid methylation

	Phospholipid methylation (pmol/10^6 cells/30 min)		Testosterone production (ng/10^6 cells/3 h)	
	−hCG	+hCG	−hCG	+hCG
Control	0.23 ± 0.01	0.80 ± 0.1*	2.9 ± 0.4	25.3 ± 2*
LHRH	0.21 ± 0.08	0.85 ± 0.02*	2.5 ± 0.5	12.8 ± 1.7*†
AVP	0.21 ± 0.05	0.79 ± 0.06*	3.0 ± 0.5	14.6 ± 2.5*†

Leydig cells were preincubated for 10 minutes with 10 μCi [*methyl*-^{3}H]methionine (21.3 μM) and then, medium (controls), hCG (670 ng/ml), LHRH (0.8 nM), LHRH (0.8 nM) + hCG (670 ng/ml), AVP (0.1 uM), AVP (0.1 uM) + hCG (670 ng/ml) were added. The incubation was continued for 30 minutes and then phospholipids were extracted as indicated in Experimental. For testosterone production, interstitial cells (approximately 3 × 10^6) were incubated with the indicated hormones for 3 hours. Testosterone concentration was determined by RIA as described in Experimental

* $P < 0.005$ compared with the value without hCG (Student's *t* test).

† $P < 0.005$ compared with its respective control with hCG (Student's *t* test).

Effect of dbcAMP, cholera toxin, and forskolin on phospholipid methylation of Leydig cells

The incubation of Leydig cells with 1 mM dbcAMP in the presence of 1 mM MIX for 30 minutes did not stimulate PL methylation. Similar results were obtained when Leydig cells were incubated in the presence of 10 μg/ml cholera toxin or 5 μM forskolin to directly activate adenylate cyclase (Figure 4).

Effect of the isolated hCG subunits (α and β) and hybrid hCG in Leydig cell phospholipid methylation

As illustrated in Figure 5, hCGβ subunit (34 μg/ml) considerably stimulated phospholipid methylation

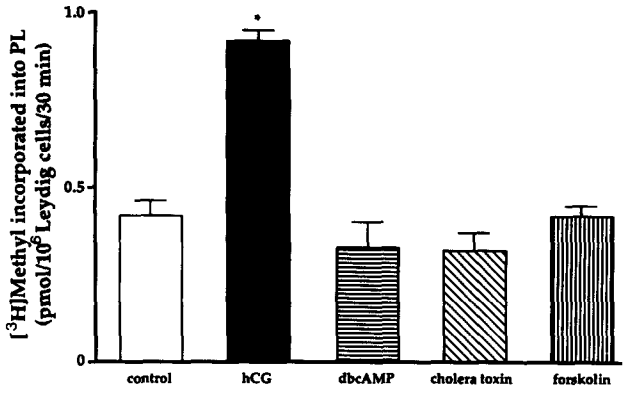

Figure 4 Effect of hCG (670 ng/ml), dbcAMP (1 mM) plus MIX (1 mM), cholera toxin (10 μg/ml), and forskolin (5 μM) on phospholipid methylation. Results are expressed as the mean ± SD of three experiments run in triplicate. *$P < 0.005$ compared with the control (Student's *t* test).

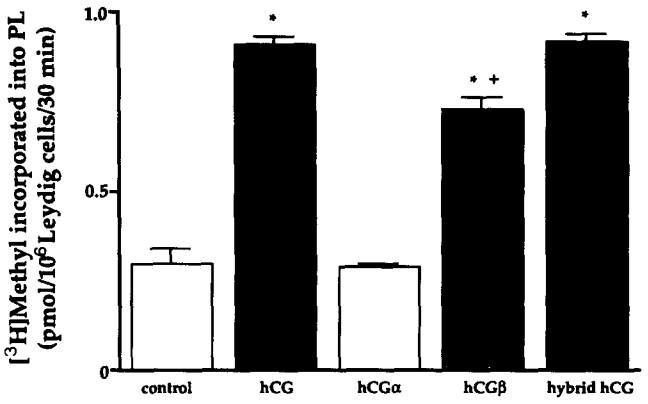

Figure 5 Effect of hCG (670 ng/ml), hCGβ subunit (34 μg/ml), hCGα subunit (34 μg/ml), and hybrid hCG (1 IU) on phospholipid methylation. Results are expressed as the mean ± SD of three experiments run in triplicate. *$P < 0.005$ compared with the control, +$P < 0.02$ compared with the hCG value (Student's *t* test).

(233% ± 9%, mean ± SD, n = 3, $P < 0.005$) compared with control Leydig cells. This hCGβ stimulatory effect was lower than that obtained with native hCG (297% ± 4%, mean ± SD, n = 3, $P < 0.005$). The hCGα subunit (34 μg/ml) did not stimulate phospholipid methylation. When reconstituted, the hybrid molecule stimulated the incorporation of [^{3}H]methyl groups to the same level as did the native hormone. Additional experiments demonstrated that neither hCGβ or hCGα subunit were able to stimulate testosterone production. However, the reconstituted hybrid molecule stimulated Leydig cell testosterone production to the same level as obtained with native hCG.

Discussion

In the present report, we present evidence that LH/hCG can stimulate phospholipid methylation in intact Leydig cells. This effect is time and dose dependent and is not mediated by cAMP. The stimulatory effect of hCG is specific, because LHRH and AVP, two known regulators of the Leydig cell steroidogenesis, did not produce any effect on phospholipid methylation. Our results do not agree with those previously reported by Moger,[13] where LH had no effect on the rate of phospholipid methylation by intact Leydig cells. This discrepancy could be due to the different total methionine concentration used. In our experimental system, a 20-μM methionine concentration was used, which is similar to the reported intracellular methionine concentration in somatic cells,[19] whereas in Moger's system,[13] 0.2 μM concentration was used. Thus, the intracellular SAM concentration may not have been high enough to detect any change in phospholipid methylation. The K_m for SAM reported by Nieto and Catt[4] and by us[15] is 4 and 9 μM, respectively.

Dose-response curves of hCG-stimulated phospholipid methylation and testosterone production showed a close correlation, although different sensitivities to hCG concentration were observed. At a low hCG dose,

steroidogenesis occurs even at low phospholipid methylation levels, indicating a dissociation between both processes. These results suggest that hCG-stimulated phospholipid methylation is mainly dependent on the number of occupied receptors. Alternatively, phospholipid methylation measurement may not be sensitive enough to detect low PL changes occurring within localized membrane microdomains of the cell.

No stimulatory effect on phospholipid methylation by other adenylate cyclase stimulators and cAMP analogs was observed. These observations demonstrated that the LH/hCG-stimulated phospholipid methylation in intact Leydig cells was not mediated by cAMP. However, Nieto and Catt[4] have previously found that cAMP or cholera toxin stimulated PLMT activity twofold in disrupted Leydig cells. Thus, there was no correlation between phospholipid methylation results in intact cells and PLMT activity assayed in vitro. This absence of correlation was not totally unexpected, because no correlation has been found in several somatic cells.[5–7] The increase in PLMT activity observed with cAMP could be due to the loss of an inhibitory factor during the homogenization of Leydig cells. This inhibitory activity has been found in rat liver cells and is probably localized on the inner face of the plasma membrane.[20] It has also been shown that although purified liver PLMT is susceptible to phosphorylation, this modification did not significantly affect the enzyme activity.[21]

It is known that the beta subunit is specific for each gonadotropin and confers immunological and biological specificity on the individual hormones.[22] Moreover, the isolated subunits are generally inactive, and full biological activity is restored only after their recombination.[23] The stimulatory effect observed with hCGβ subunit strongly suggests that hCGβ subunit was the main subunit involved in phospholipid methylation activation. A contamination with the heterodimer or the complementary subunit has been shown to be negligible.[24] In addition, we did not find steroidogenic activity when both subunits were separately tested (data not shown). The reconstituted hybrid molecule stimulated the incorporation of [^{3}H]methyl groups up to the same level as the native hormone did. Therefore, these results constitute the first observation that one individual hCG subunit can generate a marked biological response in the rat Leydig cell.

The stimulatory hCG effect on phospholipid methylation should be specific for gonadotropins, because hormones such as LHRH and AVP did not affect these reactions. Previous studies with phospholipid methylation inhibitors that decreased LH-stimulated steroid production suggested that phospholipid methylation reactions are involved in LH/hCG-stimulated steroidogenesis.[3,8] Taken together, these results demonstrate a specific involvement of LH/hCG on Leydig cell phospholipid methylation, and probably on LH/hCG-stimulated steroidogenesis through a mechanism involving phospholipid methylation activation.

It has been postulated that phospholipid methylation facilitates the activation of adenylate cyclase.[2] In Leydig cells, phospholipid methylation inhibitors decreased the hCG-stimulated cAMP production.[15] However, that effect was not important enough to account for the observed decrease in testosterone production.[8]

In several somatic cells, a direct relationship between phospholipid methylation and ion influx has been observed.[2,25] Therefore, hCG-stimulated phospholipid methylation could be related to physicochemical changes occurring at the plasma membrane level, which in turn may modulate ion fluxes. In this regard, Ca^{+2} influx has been demonstrated to be necessary for the hCG-stimulated testosterone production in rat Leydig cell.[26]

Results reported here, in addition to those of Nieto and Catt,[4] give further support to a role for LH/hCG as specific stimulator of phospholipid methylation in Leydig cells. Our results also suggest that phospholipid methylation activation is not a general signal transduction mechanism because LHRH and AVP, two hormones with specific receptors on the Leydig cell surface, did not stimulate phospholipid methylation. In addition, the LH/hCG stimulatory effect is mainly related to the hCGβ subunit, an important finding that implies a biological function for this beta subunit. Therefore, a new role for free β subunit may be stated. We conclude that LH/hCG-stimulated phospholipid methylation, required for steroidogenesis, involves hormone receptor interaction through the hCGβ subunit and does not require cAMP.

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