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Gonadotropin-Releasing Hormone Signaling Pathways in an Experimental Ovarian Tumor

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Previous results showed that GnRH signaling is altered in cells from rat luteinized ovarian tumors (tumor group) because it did not activate the phospholipase C pathway, in contrast to control ovarian cells from superovulated prepubertal rats (SPO). In the present work, alternate GnRH-induced second messengers such as phospholipase A_2 and phospholipase D activation, cAMP production, ERK1/2 phosphorylation, and the presence of G proteins were evaluated to determine GnRH mechanism of action in tumor cells. G proteins examined were present in both cell types. Buserelin, a GnRH agonist, (1, 10, and 100 ng/ml) increased phosphatidylethanol in SPO, indicating phospholipase D activation. Only 100 ng/ml buserelin induced a significant response in the tumor group. Buserelin (100 ng/ml) increased ^{3}H-arachidonic acid in culture media in SPO, indicating phospholipase A_2 activation; no effect was observed in the tumor group. Buserelin (100 and 1000 ng/ml) induced pertussis toxin-insensitive cAMP increases in both cell types, with similar potencies. In the tumor group, buserelin (100 ng/ml) inhibited human chorionic gonadotropin-induced cAMP and progesterone; this effect was protein kinase C (PKC) dependent (inhibited by GF109203X, a PKC inhibitor). Buserelin (100 and 1000 ng/ml) induced ERK1/2 phosphorylation in both cell kinds. Buserelin-induced ERK1/2 activation was $G_{i/0}$ independent and PKC dependent. Only in the tumor group, buserelin-induced ERK1/2 activation was cAMP dependent (abolished by SQ 22536, the adenylyl cyclase inhibitor). Furthermore, dibutyryl cAMP-induced ERK1/2 activation in the tumor group was PKC dependent (inhibited by GF109203X). In conclusion, activation of phospholipases in tumor cells does not seem to mediate GnRH effects. GnRH signaling seems to involve adenylyl cyclase activation, PKC stimulation, and ERK1/2 phosphorylation. (*Endocrinology* 144: 2957–2966, 2003)

A LOCAL GnRH system has been widely described both in the normal and tumoral ovary. The presence of the GnRH peptide and receptor mRNAs and corresponding proteins has been demonstrated in these tissues (1–8). Its role as an autocrine/paracrine regulator in the ovary is endorsed by a multitude of effects attributed to GnRH receptor-mediated signaling, which range from basal and gonadotropin-stimulated steroidogenesis modulation to induction of transcription of several genes involved in follicular maturation, ovulation, and atresia (9, 10). Furthermore, GnRH has been shown to have direct growth-inhibitory effects in hormone-sensitive tumors (11–13), including carcinoma of the ovary (14–17). In previous works, we had demonstrated that a GnRH superactive analog inhibited the development of an experimental intrasplenic, highly luteinized ovarian tumor (luteoma) by inhibiting gonadotropin secretion (18). A direct action on the tumor was also suggested by GnRH receptor presence in the tumor and GnRH inhibitory action on tumor hormone production (19). When analyzing the mechanism of action behind these effects, we demonstrated that the classical GnRH signaling pathway, phospholipase C (PLC) activation, appeared to be inactive because no increase in inositol phosphates (IPs) or intracellular calcium concentration was induced by the agonist (6). This lack of PLC activation under GnRH stimulation was also observed in other GnRH receptor-positive endocrine tumors (12, 17).

In addition to the classical GnRH transduction pathway, involving $G_{q/11}$ stimulation, which activates PLCβ, generating the second messengers inositol 1,4,5-triphosphate and calcium as well as early diacylglycerol (DAG) required for conventional protein kinase C (PKC) activation, other phospholipases have also been described to be activated by GnRH. After a short lag time (1–2 min), GnRH can activate phospholipase A_2 (PLA_2) and phospholipase D (PLD), which may provide late DAG for activation of Ca^{2+}-independent, novel PKC isoforms (20, 21). Moreover, arachidonic, oleic, linoleic, and linolenic acids can activate specific PKC isoforms with no requirements for other cofactors or support activation of PKCs in the presence of cofactors such as Ca^{2+}, DAG, or phosphatidylserine (21). Thus, sequential activation of cellular phospholipases by GnRH will provide lipid-derived PKC activators in a time-dependent fashion that may elicit various physiological tasks. Furthermore, PKC-dependent and independent GnRH activation of MAPK cascades have also been demonstrated, which may mediate nuclear transcriptional regulation by the activation of c-fos and c-jun transcription factors among others (22). This has

Abbreviations: BIC, Sodium bicarbonate; DAG, diacylglycerol; FAF, fatty acid free; ^{3}H-AA, ^{3}H-arachidonic acid; hCG, human chorionic gonadotropin; IBMX, 3-isobutyl-1-methylxanthine; IP, inositol phosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; PE, phosphatidylethanol; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; rt, room temperature; SPO, superovulated rat ovaries.

been demonstrated in pituitary cells (23–27) as well as in ovarian and testis cells (28–31). In addition, activation of tyrosine kinases by GnRH has also been demonstrated in αT3-1 cells, a gonadotroph cell line (27).

Evidence from toxin and second messenger studies indicated that multiple G proteins mediate the actions of the GnRH receptor (10, 32). Recently, using GnRH-stimulation of G protein palmitoylation, Stanislaus and colleagues (33, 34) demonstrated that GnRH receptors couple to $G_{q/11}$, $G_{i\alpha}$, and $G_{s\alpha}$ proteins in rat gonadotropes. Coupling to Gi was also demonstrated in reproductive tract tumors by different methodologies (12, 16, 33–35). As discussed above, GnRH receptors are coupled to various G proteins, and changes in receptor number may result in differential coupling to these proteins, enabling the activation of different signal transduction pathways and ultimately achieving different physiological end points. An example of this was demonstrated in GH_3 cells expressing GnRH receptors: although GnRH-stimulated cAMP production was not modified by changes in receptor number/cell, GnRH-stimulated IP production increased when receptor number augmented (36).

In view of the complexity of the GnRH signaling system, we decided to study the alternative pathways activated by GnRH to induce its effects in luteoma cells. Activation of PLD, PLA_2, adenylyl cyclase, and MAPK were evaluated in luteoma and control luteinized cells under GnRH stimulus as well as the expression of G proteins in both cell kinds.

Materials and Methods

Animals

Adult female virgin Sprague Dawley rats (200–250 g) from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room, with lights on from 0700–1900 h. They were given free access to laboratory chow and tap water. At the end of experimental procedures, animals were killed by decapitation according to protocols for animal use, approved by the institutional animal care and use committee (Instituto de Biología y Medicina Experimental-Consejo Nacional de Investigaciones Científicas y Técnicas) that follows NIH guidelines.

For the tumor-bearing group, 50 animals were bilaterally ovariectomized and one ovary was implanted into the spleen 6–8 wk before the experiments (luteoma), as previously described (18, 19, 37).

Control animals (81) were 23- to 25-d-old female rats, injected with 25 IU pregnant mare's serum gonadotropin (Novormon, Syntex, Buenos Aires, Argentina) and 25 IU human chorionic gonadotropin (hCG) (Endocorion, Elea, Buenos Aires, Argentina) 48 h later. These animals were used 5 d after hCG injection (superovulated rat ovaries, SPO).

Cell culture

Cells from ovarian tumors, as well as from 23- to 25-d-old SPO, were isolated with collagenase (Life Technologies, Inc., Grand Island, NY), as described previously (18, 19). Each tumor yielded approximately 8×10^6 cells, and each SPO ovary yielded approximately 2×10^6 cells. Briefly, cells were plated in plastic 24-well culture dishes, coated with 0.5 mg/ml collagen (Sigma, St. Louis, MO) and incubated in sodium bicarbonate (BIC)-fetal calf serum: DMEM-F12 (Life Technologies, Inc.) with 2.2 g/liter sodium bicarbonate, 10% fetal calf serum (Life Technologies, Inc.), Fungizone (Life Technologies, Inc.), and gentamicin (Life Technologies, Inc.). After 24 h plating media were replaced. After 7 d in culture, cells were washed once with serum-free BSA-supplemented (Sigma) medium (DMEM-F12 with 2.2 g/liter sodium bicarbonate, 0.1% BSA: BIC-BSA) and then incubated with the corresponding stimuli in this medium, for the indicated time periods.

G protein expression

To assess the expression of G proteins, 8-d-old cultures of luteoma and SPO cells (700,000 cell/well) were washed with ice-cold PBS and 50 μl sample buffer (125 mM Tris base, 1% sodium dodecyl sulfate, 0.1% bromophenol blue, 5% glycerol, 25 mM dithiothreitol) were added. Cells were scraped and lysates transferred to tubes (Eppendorf, Hamburg, Germany), heated to 100 C for 5 min, centrifuged at $10,000 \times g$ for 5 min and kept at −70 C. On the day of the electrophoresis, 20 μl cell lysates were run on 15% SDS-PAGE gels and electrotransfer to a nitrocellulose membrane. Membranes were blocked with 5% BSA-TTBS buffer (0.1% Tween 20, 10 mM Tris base, 0.07 M NaCl, pH 8) 1 h at room temperature (rt) and incubated with G protein antibodies raised against $G_{\alpha q/11}$ (1:1000) kindly donated by Dr. P. Michael Conn and $G_{\alpha 0}$ (1:800), $G_{\alpha i\text{-}1}$ (1:1000, which also cross-reacts with $G_{\alpha i\text{-}2}$ and $G_{\alpha i-3}$) and $G_{\alpha s}$ (1:1600) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at rt. After washing, the signals were detected with a horseradish peroxidase-conjugated antirabbit secondary antibody (1:2000, 1 h at rt) and visualized by chemiluminescent detection. Immunoblot images are shown. Experiments were repeated three times.

PLD activity

PLD activity was determined as described by Liscovich and Amsterdam (38). Briefly, luteoma and SPO cells (400,000 cells/well) were preincubated with oleic acid [9,10-^{3}H(N)] (2 μCi/well, NEN Life Science Products, Boston, MA) for 24 h in BIC-0.1% fatty acid free (FAF)-BSA (BIC-FAF-BSA) (Sigma). ^{3}H-Oleic acid incorporations were 63.0% ± 1.1% in tumor cells and 75.5% ± 0.9% in SPO cells. Thereafter, cells were washed three times with BIC-FAF-BSA. Medium was changed to BIC-FAF-BSA-0.5% ethanol and incubated for 60 min in the presence of buserelin, a GnRH superactive agonist ([D-Ser(-tBu)^6-des-Gly^{10}]-GnRH-*N*-ethylamide, a gift from Hoechst, Buenos Aires, 1, 10, or 100 ng/ml) or phorbol 12-myristate 13-acetate (PMA, 100 mM, Sigma) as a positive control. To terminate incubations, the plates were placed on ice and rinsed twice with ice-cold PBS. Cold methanol (1 ml) was added to each well, cells were scraped, and the methanolic suspension was transferred to glass extraction tubes. Chloroform (1 ml) and 1 ml of a mixture of 1 N HCl and 1 mM EGTA (Sigma) were added, and the tubes were vortexed and centrifuged (10 min at $360 \times g$). The lower chloroform phase containing the extracted lipids was collected, dried, and kept at 4 C for analysis. ^{3}H-Phosphatidylethanol, produced by PLD phosphatidyl transferase activity when ethanol acts as the phosphatidyl group acceptor, and ^{3}H-phosphatidic acid were separated from the other lipids and from each other by thin-layer chromatography (LK6D silica gel plates, Whatman, Clifton, NJ) with the organic phase of a mixture of ethyl acetate/2,2,4-trimethyl pentane/acetic acid/water (13:2:3:10). The regions comigrating with the phosphatidylethanol (PE) and phosphatidic acid (PA) standards (ICN Biochemicals, Irvine, CA) in the plates were scraped and counted. Retention factors for PE and PA acid were 0.65 ± 0.03 and 0.55 ± 0.03, respectively. Results are expressed relative to ^{3}H-oleic acid incorporation in each culture. Experiments were repeated four times.

PLA_2 activity

PLA_2 activity was determined as described by Watanabe *et al.* (39) with minor modifications. After 48 h in culture, cells (400,000 cells/well in 24-well culture dishes) were incubated with arachidonic acid [5,6,8,9,11,1,2,14,15-^{3}H(N)] (0.5 μCi/ml per well, NEN Life Science Products) for 24 h. ^{3}H-Arachidonic acid (^{3}H-AA) incorporations were 54.5% ± 7.5% in tumor group cells and 69.5% ± 2.5% in SPO cells. Prelabeled cells were washed three times with BIC-FAF-BSA and then preincubated with the same buffer for 45 min. Thereafter cells were incubated in the presence or absence of quinacrine (10 ml/well, 200 μM, ICN) for 15 min. Stimuli were added (buserelin: 100 ng/ml or prostaglandin F 2α: 1.10^{-5} M) for 10 min. Dishes were then placed on ice, and media were collected and counted by liquid scintillation. Results are expressed relative to ^{3}H-AA incorporation in each culture. To determine that the radioactivity in the medium corresponded to arachidonic acid, organic extracts of culture media were injected into a reverse-phase column (Microsorb-MV C18) and analyzed by HPLC with a solvent gradient that ranged from acetonitrile:water:acetic acid (50:50:0.1) to acetonitrile:acetic acid

(100:0.1) for 40 min and was followed by an isocratic phase with the last solvent mixture for 10 min as described by Omata *et al.* (40). The elution time of ^{3}H-AA standard coincided with the peak in the samples assayed, confirming the identity of the radioactive compound in the culture media. Experiments were repeated four times.

Intracellular cAMP measurement

After a preincubation of 24 h with pertussis toxin (PTX, 150 ng/ml, Sigma) in BIC-FCS or medium alone (control), luteoma and SPO cells (400,000 cells/well) were washed with BIC-BSA and incubated with buserelin (100 or 1000 ng/ml,), hCG (National Institute of Diabetes and Digestive and Kidney Diseases, 1×10^{-9} M) or forskolin (Sigma, 5 μM) for 30 min in the presence of 3-isobutyl-1-methylxanthine (IBMX) (Sigma, 0.1 mM). After incubations, cells were placed on ice and washed with BIC-BSA, 0.7 ml cold ethanol was added to each well, cells were scraped and transferred to tubes (Eppendorf), heated for 5 min at 95 C, and centrifuged at 9400 $\times$ *g*. Supernatants were dried and kept at −20 C. Finally the extracts were resuspended in cAMP RIA buffer. Intracellular cAMP was measured by RIA as described previously (41) using the antibody provided by NIDDKD. Assay sensitivity was 20 fmol/400,000 cells. Intra- and interassay coefficients of variation were 8.1% and 10.5%, respectively. Experiments were repeated four times. Results are expressed as percentage of increase over basal levels taken as 100%.

Extracellular signal-regulated kinase determination by Western blots

Luteoma and SPO cells (400,000 cells/well) were incubated for 10 min (26) with buserelin, (100 or 1000 ng/ml) or PMA (1 μM) alone or in combination with the following pretreatments: PTX (150 ng/ml, 24 h before the experiment to ADP ribosylate Gi/0), PMA (1 μM, 24 h before the experiment to deplete PKC), PD 98059 [Sigma, 50 μM, 1 h before the experiment to inhibit MEK, the MAPK phosphorylating enzyme]. In a second set of experiments, cells were treated with medium or buserelin (1 ng/ml) alone or in combination with the following pretreatments: SQ 22536 (ICN, an adenylyl cyclase inhibitor, 0.5 mM, 30 min before stimuli) or SQ 22536 (0.5 mM) in combination with PMA (1 μM, 24 h before the experiment). In another set of experiments, the tumor group cells were incubated for 10 min with dibutyryl cAMP (1 mM) in the presence or absence of bisindolylmaleimide I (GF 109203X, ICN Biomedicals, Inc., Aurora, OH, a PKC inhibitor, 25 μM, 30-min preincubation before the stimuli were added) .

After the incubations, cells were washed with ice-cold PBS and 50 μl sample buffer were added. Cells were scraped, lysates were transferred to tubes (Eppendorf) and kept at −70 C. On the day of the electrophoresis, cell lysates were heated at 100 C for 5 min, centrifuged at 10,000 $\times$ *g* for 5 min; 20-μl lysates were then run on 15% SDS-PAGE gels and electrotransferred to a nitrocellulose membrane (Amersham Pharmacia, Uppsala, Sweden). Membranes were blocked for 2 h at rt with 3% fat-free milk-TTBS. Phospho-specific extracellular signal-regulated kinase antibody (1:2000), which detects 44 MAPK (ERK 1) and 42 MAPK (ERK 2) only when phosphorylated at Thr (202) and Tyr (204) (P-ERK), was used to measure MAPK activation (New England Biolabs, Inc., Beverly, MA). Alternatively, the membranes were probed with a rabbit polyclonal antibody for 44/42 MAPK (1:1200), which detects total MAPK (ERK), independent of the phosphorylation state (New England Biolabs, Inc.). Incubation with first antibodies was done for 1 h at rt. After washing, the signals were detected with horseradish peroxidase-conjugated secondary antibody (1:2000, 1 h rt) and visualized by chemiluminescent detection. Quantification of immunoblots was performed with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). P-ERK levels were standardized against the levels of ERK per sample. Experiments were repeated three to five times.

Progesterone determinations

Progesterone was determined in culture media of the tumor group cells (400,000 cells/well) stimulated with hCG (1×10^{-9} M) in the presence or absence of buserelin (100 ng/ml) for 24 h and with or without preincubations with PTX (150 ng/ml, 24-h preincubation before the stimuli were added) or GF 109203X (25 μM, 30-min preincubation before the stimuli were added). Progesterone was determined by RIA using a specific antiserum kindly provided by Dr. G. D. Niswender. Labeled hormones were purchased from NEN Life Science Products. Assay sensitivity for progesterone was 6.25 pg and intra- and interassay coefficients of variation were 7.5% and 11.9%, respectively. Experiments were repeated three times.

Statistical analysis

All experiments were run in replicates and repeated three to five times. Results are expressed as mean ± SE. The data were analyzed by one-way or two-way ANOVA for paired samples, followed by *t*-Newman-Keuls multiple comparisons test. When only two groups were compared, *t* test was used. In all cases results were considered significant at $P < 0.05$.

Results

G protein expression in ovarian tumor and control cells

As in previous works in which we demonstrated that tumor cells differed from control luteal cells in GnRH signaling, our aim was to determine whether this could be due to differences in G protein expression. Here we demonstrate that both cell kinds express all the G proteins investigated, $G_{\alpha i}$, $G_{\alpha 0}$, $G_{\alpha s}$, and $G_{\alpha q/11}$, at the expected molecular weights (Fig. 1), indicating that the difference in response to GnRH between tumor and control cells was not due to lack of some kind of G protein presence in the cells.

Buserelin-induced PLD activation in ovarian tumor and control cells

The activation of PLD, measured as the increase in intracellular PE and PA relative to ^{3}H-oleic acid incorporation in response to different stimuli, was determined in both tumor and SPO cells. ^{3}H-oleic acid incorporation was 63.0% ± 1.1% in tumor cells and 75.5% ± 0.9% in SPO cells, the difference

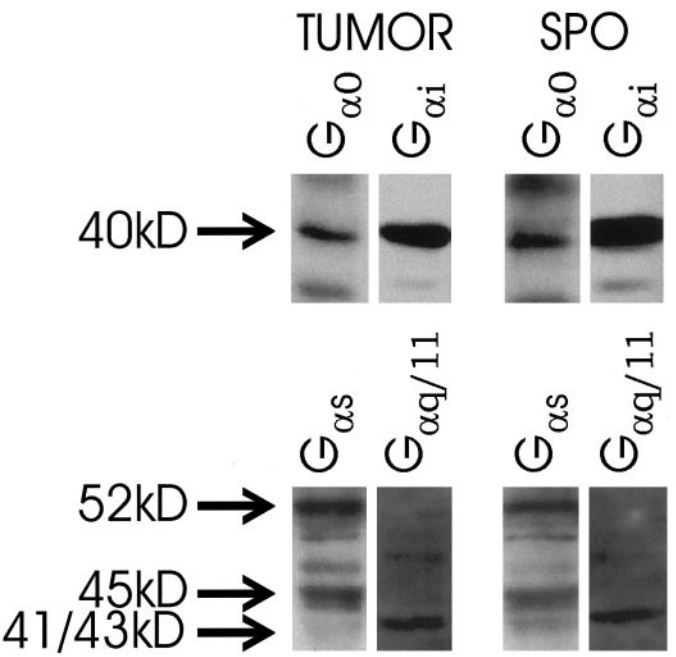

FIG. 1. G protein expression in tumor and SPO cells. G proteins were determined by Western blots with specific antisera. *Arrows* indicate the molecular masses at which the specific bands were expressed. Experiments were repeated three times.

being statistically significant ($P < 0.001$). In basal conditions, tumor cells showed significantly lower PE levels than SPO cells [tumor group (^{3}H-PE/^{3}H-OA incorporated): 1.78 ± 0.05 *vs.* SPO (^{3}H-PE/^{3}H-OA incorporated): 2.77 ± 0.25, $P < 0.01$, n = 4]. In addition, although SPO cells showed a concentration-dependent increase in PE in the presence of buserelin (1, 10, or 100 ng/ml) after 60-min incubation, achieving statistical significance from 1 ng/ml onward (Fig. 2, *right panel*) in tumor cells only, the highest concentration stimulated PE increase (Fig. 2, *left panel*) and then to a lesser degree than in SPO cells [buserelin-induced PE (fold increase), tumor group: 1.23 ± 0.05 *vs.* SPO: 1.510 ± 0.09, $P < 0.03$, n = 4]. The response to PMA (0.5 μM), a stimulus that is known to activate PLD, was also significantly more intense in SPO than in tumor cells [PMA-induced PE (fold increase), tumor group: 3.75 ± 0.77 *vs.* SPO: 7.77 ± 0.67, $P < 0.01$, n = 4].

In addition, buserelin (100 ng/ml) and PMA (0.5 μM) stimulated significant increases in PA in both cell types (not shown).

Buserelin-induced PLA_2 activation in ovarian tumor and control cells

The activation of PLA_2, measured as the increase of free ^{3}H-AA in the culture medium relative to ^{3}H-AA incorporation into each culture after 10 min of cell stimulation with either buserelin (100 ng/ml) or PGF2α (1.10^{-5} M, used as a control) was determined. ^{3}H-AA incorporation into tumor cells ranged from 31–67%, with a mean incorporation of 54.5 ± 7.5% although in SPO cells it ranged from 64–75% with a mean incorporation of 69.5 ± 2.5%, the difference being statistically significant ($P < 0.04$).

SPO cells responded significantly to both stimuli with an increase in ^{3}H-AA levels in the culture media (Fig 3, *right panel*). In contrast, no increase in ^{3}H-AA in the media of tumor cells was evident (Fig 3, *left panel*). In the presence of 200 μM quinacrine, a PLA_2 inhibitor, a significant decrease in basal ^{3}H-AA levels was observed in SPO cells. In addition, the buserelin and PGF2α-stimulating effects were completely

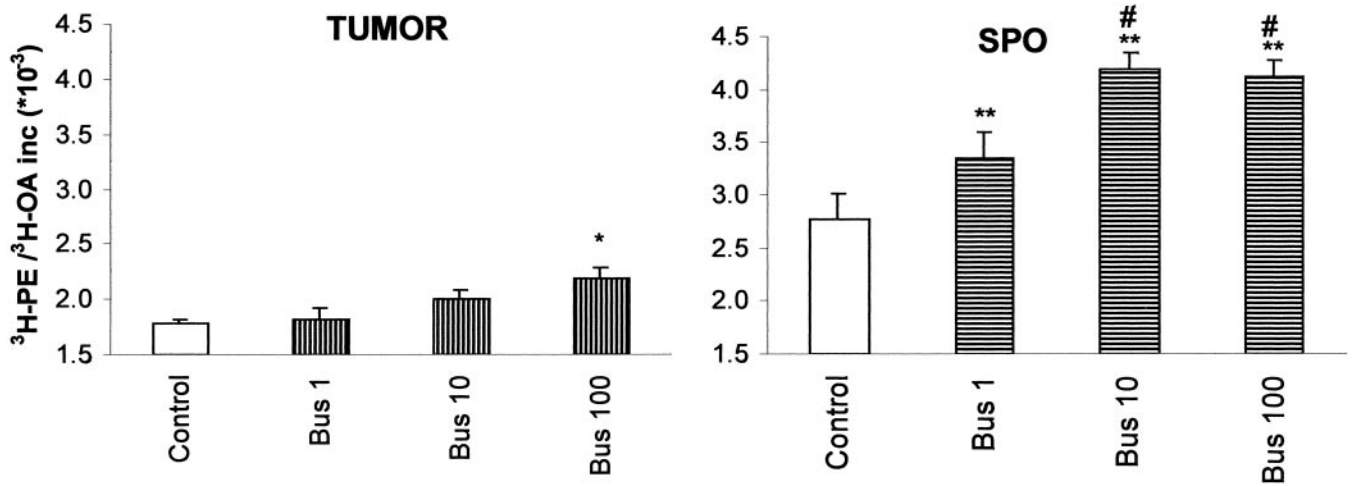

FIG. 2. Buserelin (Bus, 1, 10, or 100 ng/ml) dose-response activation of PLD, measured as the ratio of ^{3}H-PE formed (cpm) after 60-min stimulation over ^{3}H-oleic acid incorporated into the cells (^{3}H-OA, cpm) in tumor and SPO cells. Experiments were repeated four times. *, Significantly different from control. #, Significantly different from Bus 1. $P \leq 0.05$.

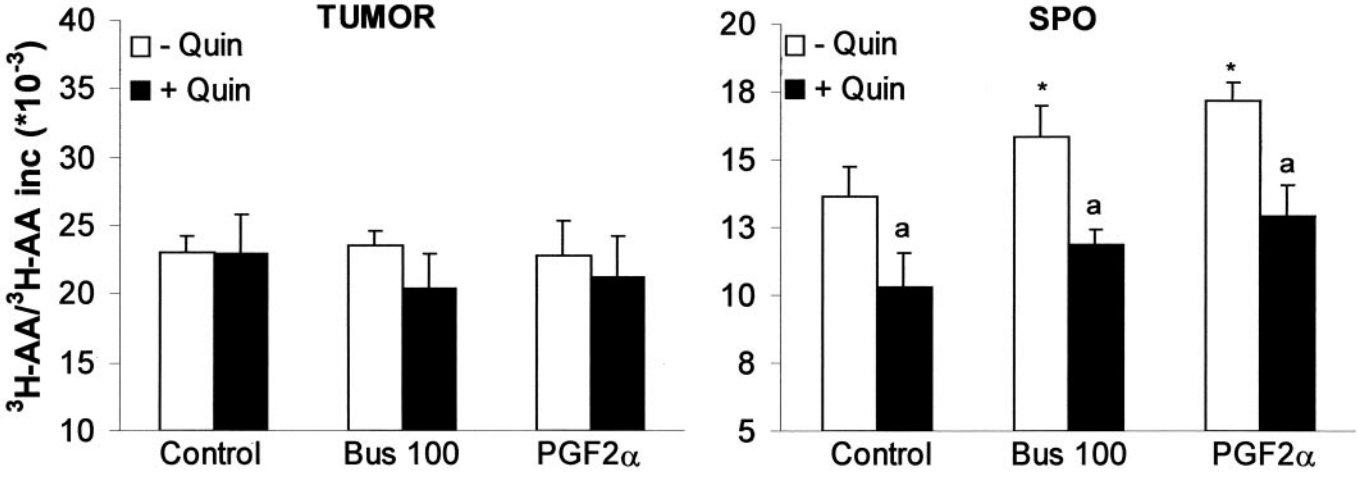

FIG. 3. Effect of buserelin (Bus, 100 ng/ml) and PGF2α (1×10^{-5} M) on PLA_2 activation measured as the ratio of ^{3}H-AA liberated into the media (cpm) after 10-min stimulation over ^{3}H-AA incorporated into the cells (cpm). Experiments were performed in the presence or absence of quinacrine, the PLA_2 inhibitor (Quin, 200 μM) in tumor and SPO cells. Experiments were repeated four times. *, Significantly different from control. a, Significantly different from cells without quinacrine. $P \leq 0.05$.

abolished, indicating the specificity of the response. In tumor cells, no significant variations in ^{3}H-AA levels, in either basal or stimulated conditions, were observed in the presence of the inhibitor.

Buserelin effect on cAMP production in ovarian tumor and control cells

The cAMP levels were determined in cell cultures in the presence of 0.1 mM IBMX to inhibit cAMP degradation by phosphodiesterases. Basal intracellular cAMP levels, when corrected relative to DNA content, were approximately 6 times higher in control luteinized cells (SPO) that tumor cells [cAMP (fmol/μg DNA), tumor cells: 18.4 ± 1.2 *vs.* SPO cells: 114.5 ± 1.0, $P < 0.01$, n = 6]. To analyze the capacity of each cell type to respond to different stimuli, cAMP levels were expressed as the percentage of increase with regard to basal levels. Forskolin, an adenylyl cyclase stimulator, was used as a positive control, and the response of both cells types to this stimulus was similar (not shown). Buserelin (100 and 1000 ng/ml) induced an increase in cAMP production in both cell types, but although tumor cells responded to both concentrations assayed, SPO cells responded only to the lower one and to a similar degree as tumor cells (Fig. 4, *upper left* and *right panels*). The effect of hCG was tested either alone or in combination with buserelin on both cell types. This gonadotropin significantly stimulated cAMP production in both cell types (Fig. 4, *middle left* and *right panels*). The hCG induced significantly higher cAMP levels in tumor cells than in SPO cells [cAMP (fold response), tumor-hCG: 3 ± 0.7 *vs.* SPO-hCG: 1.7 ± 0.2, $P < 0.05$, n = 6]. In addition, although in tumor cells the combination of hCG and buserelin inhibited the hCG-induced cAMP production to levels observed with buserelin alone, in SPO cells the combination of drugs induced cAMP levels similar to those observed with either hCG or buserelin (Fig. 4, *middle left* and *right panels*). To determine whether the buserelin effect on cAMP production involved a G protein of the i/0 kind, cells were pretreated overnight with PTX and then subjected to the buserelin stimulus. The effect of buserelin was not altered by PTX preincubation in either cell kind, but Gi/0 inactivation induced a significant increase in basal cAMP levels only in tumor cells (Fig. 4, *lower left* and *right panels*).

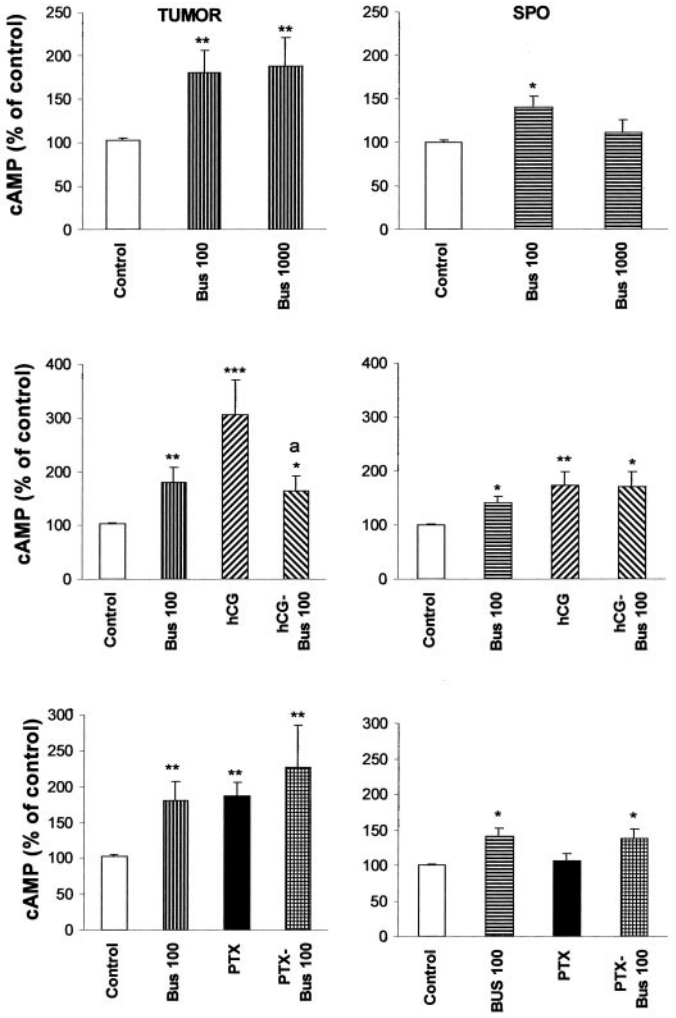

FIG. 4. The cAMP (percent of control) in tumor and SPO cells treated with different agents for 30 min in the presence of IBMX. *Upper panel*, Buserelin (Bus, 100 and 1000 ng/ml). *Middle panel*, Bus 100 ng/ml, hCG: 1×10^{-9} M and the combination of both. *Lower panel*, Bus (100 ng/ml), PTX (150 ng/ml, 24-h preincubation) and the combination of both. Experiments were repeated four times. *, Significantly different from control. a, Significantly different from hCG. $P \leq 0.05$.

Effect of buserelin on basal and hCG-induced progesterone secretion in tumor cells: participation of Gi/0 protein

The following experiments were performed only in tumor cells because we had previously shown that in control SPO cells buserelin does not alter hCG-induced progesterone secretion (19). In tumor cells buserelin showed a tendency to increase progesterone levels, although not attaining statistical significance; in addition, it exerted the expected inhibitory effect on hCG-stimulated progesterone levels (Fig. 5, *upper panel*). These results are in agreement with the effect of buserelin on cAMP levels (see above). To further assess the participation of Gi/0 proteins in buserelin inhibition of hCG-stimulated progesterone secretion, tumor cells were pretreated with PTX. PTX by itself significantly increased progesterone levels [Prog (pg/400,000 cells), control: 9.7 ± 1.7 *vs.* PTX: 34.1 ± 4.9, $P < 0.01$, n = 3] consistent with the above-mentioned increase in cAMP titers. The combination of PTX and hCG potentiated the progesterone-secreting effect (Fig. 5, *lower panel*). The buserelin-induced inhibition of hCG-stimulated progesterone was not altered in the presence of PTX, indicating that buserelin is not acting through Gi/0 proteins to induce this effect in tumor cells.

Participation of PKC in the inhibitory effect of buserelin on hCG-stimulated progesterone secretion in tumor cells

To evaluate whether PKC was involved in the inhibitory response of buserelin on hCG-induced progesterone secretion, tumor cells were preincubated with GF 109203X (25 mM, 30 min), a selective inhibitor of PKC. In the presence of GF 109203X, the inhibitory effect of buserelin was completely abolished, without affecting the stimulatory effect of hCG (Fig. 6), indicating that PKC is critically involved in this effect.

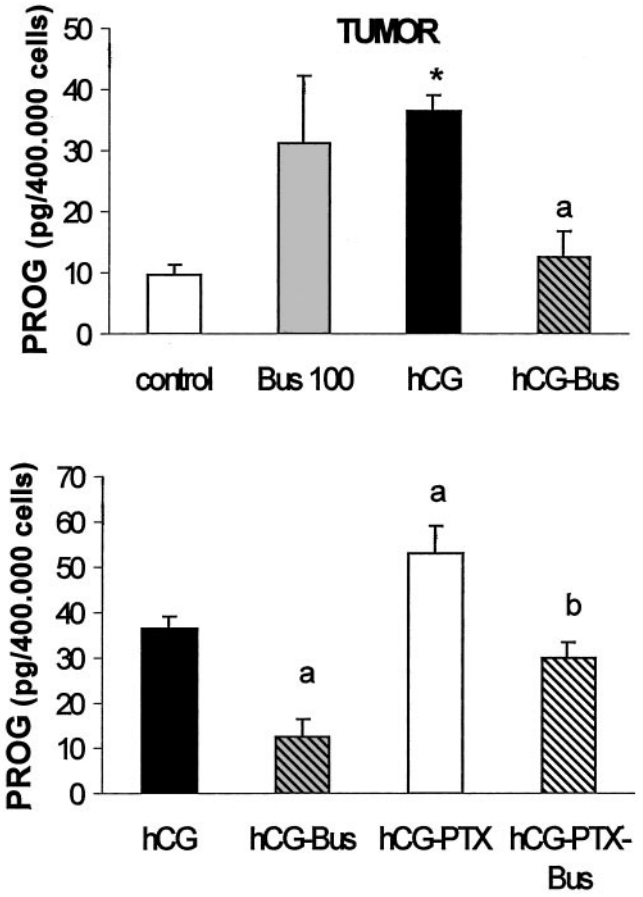

FIG. 5. Progesterone secretion into culture media in tumor cells treated with different agents for 24 h. *Upper panel*, Buserelin (Bus, 100 ng/ml), hCG (1.10^{-9} M), and the combination of both. *Lower panel*, hCG (1.10^{-9} M) alone or in combination with Bus (100 ng/ml) in the absence or presence of PTX (150 ng/ml, 24 h preincubation). *, Significantly different from control cells. a, Significantly different from hCG. b, Significantly different from hCG-PTX. Experiments were repeated three times.

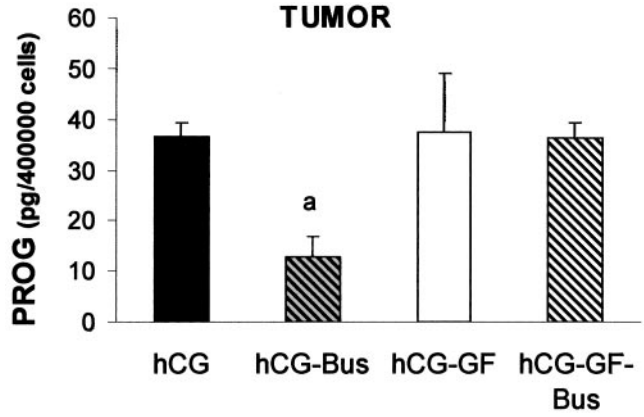

FIG. 6. Progesterone secretion into culture media in tumor cells treated with: hCG (1.10^{-9} M) alone or in combination with buserelin (Bus, 100 ng/ml) in the absence or presence of GF 109203X (25 μM), the specific inhibitor of PKC. a, Significantly different from hCG. Experiments were repeated three times.

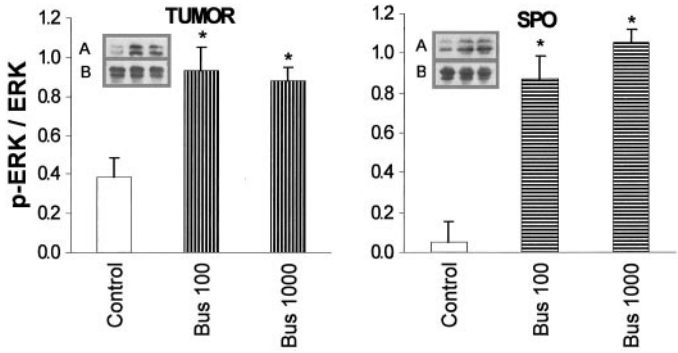

FIG. 7. *Upper panel*, Buserelin (Bus, 100 and 1000 ng/ml)-induced ERK1/2 activation in tumor and SPO cells. ERK1/2 activation was determined by Western blots with specific antisera. Quantification of immunoblots was performed by densitometry (ImageQuant software). Experiments were repeated five times. For this and the next figures, results are expressed as the mean of the ratio between phosphorylated ERK1/2 (p-ERK) and total ERK1/2 (ERK) in each well. *Inset*, Representative immunoblot, A, p-ERK, B, ERK. *Lower panel*, PD98059 inhibition of buserelin-induced ERK1/2 activation in tumor and SPO cells. Experiments were repeated five times. *, Significantly different from control. a, Significantly different from Bus.

Buserelin-induced ERK1/2 phosphorylation in ovarian tumor and control cells

Both cell types responded to buserelin with ERK1/2 activation after 10 min of incubation (Fig. 7 *left* and *right upper panels*), although the degree of stimulation was significantly higher in SPO cells (fold increase, tumor: 2.411 ± 0.614 *vs.* SPO: 19.761 ± 7.020, $P < 0.05$, n = 4). No differences were observed between the 100 and 1000 ng/ml concentrations of buserelin on ERK1/2 activation in either cell type. To asses whether buserelin-induced stimulation was a specific effect on ERK1/2 phosphorylation, buserelin was tested in combination with PD 98059 50 μM, the inhibitor of MEK (ERK kinase). In both cell kinds, PD 98059 completely abolished the activation of ERK1/2 (Fig. 7 *left* and *right lower panels*).

Effect of PMA or PTX pretreatment on buserelin-induced ERK1/2 activation in tumor and SPO cells

To test whether PKC signal transduction pathway was involved in the buserelin-induced ERK1/2 phosphorylation, cells were pretreated for 24 h with PMA (1 μM), a treatment known to deplete PKC. This pretreatment completely abolished buserelin and PMA-induced ERK1/2 phosphorylation in both cell types, indicating that it was a PKC-dependent phenomenon (Fig. 8, *left* and *right panels*). Similar results were also obtained in the presence of the PKC inhibitor, GF 109203X (not shown).

In addition, to evaluate whether buserelin-induced ERK1/2 activation was mediated by proteins of the Gi/0 kind, cells were pretreated with PTX overnight (150 ng/ml). Buserelin-induced ERK1/2 activation was not altered by PTX pretreatment in either cell kind, indicating that this kind of G protein was not involved in the response (Fig. 9, *left* and *right panels*).

Buserelin-induced ERK1/2 activation under adenylyl cyclase or adenylyl cyclase plus PKC inhibition in tumor and SPO cells

In the presence of the adenylyl cyclase inhibitor, SQ 22536, buserelin-induced ERK1/2 phosphorylation was inhibited in

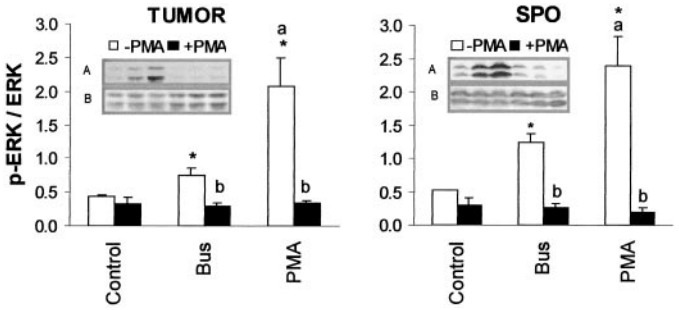

FIG. 8. Buserelin (Bus, 100 ng/ml) and PMA (1 μM)-induced ERK1/2 activation in the presence or absence of PMA pretreatment (1 μM, 24 h before the experiment) in tumor and SPO cells. Experiments were repeated four times *Inset,* Representative immunoblot; A, p-ERK; B, ERK; *open square,* without PMA pretreatment; *black square,* with PMA pretreatment. *, Significantly different from control. a, Significantly different from Bus. b, Significantly different from cells without PMA treatment.

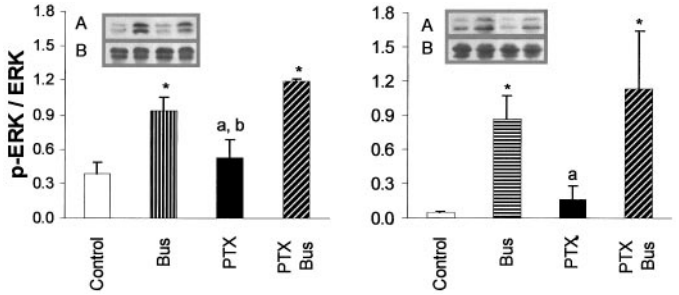

FIG. 9. Buserelin (Bus, 100 ng/ml)-induced ERK1/2 activation in tumor and SPO cells, in the presence or absence of PTX pretreatment (150 ng/ml, 24 h before the experiment). Experiments were repeated five times. *, Significantly different from control. a, Significantly different from Bus. b, Significantly different from PTX-Bus.

tumor cells (Fig. 10, *left panel*). In contrast, in control SPO cells SQ 22536 preincubation did not alter buserelin-induced ERK1/2 activation (Fig. 10, *right panel*). The combination of adenylyl cyclase and protein kinase inhibitors completely abolished buserelin-induced ERK1/2 phosphorylation in both cell kinds (Fig. 10, *left* and *right panels*).

cAMP-induced ERK1/2 phosphorylation in tumor cells

To test whether cAMP itself was able to increase ERK1/2 phosphorylation in tumor cells, these were stimulated with 1 mM dibutyryl cAMP for 10 min with or without previous preincubation with GF 109203X, the PKC inhibitor. The cAMP significantly increased ERK1/2 activation (Fig. 11). In the presence of GF 109203X, this stimulation was abolished.

Discussion

The GnRH signal transduction system has become increasingly complex involving multiple pathways. The classical pathway involves coupling to $G_{q/11}$ proteins, activation of PLCβ, and the generation of the resulting second messengers. Other signaling pathways have also been described, including stimulation of several G proteins (32), activation of PLD and PLA_2, both in pituitary (42, 43) and ovarian cells (38, 39, 44), activation of adenylyl cyclase and PKA, phosphorylation of MAPKs (10, 21, 32, 45), and activation of tyrosine

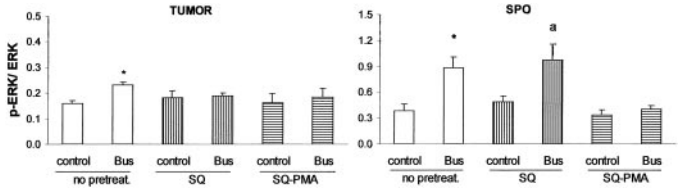

FIG. 10. Buserelin (Bus, 100 ng/ml)-induced ERK1/2 activation in tumor and SPO cells, in the presence or absence of the following pretreatments: SQ 22536, the adenylyl cyclase inhibitor (0.5 mM, 30 min before stimuli), or SQ 22536 plus PMA (1 μM, 24 h before the experiment). Experiments were repeated four times. *, Significantly different from control in cells without pretreatments. a, Significantly different from control in cells pretreated with SQ 22536.

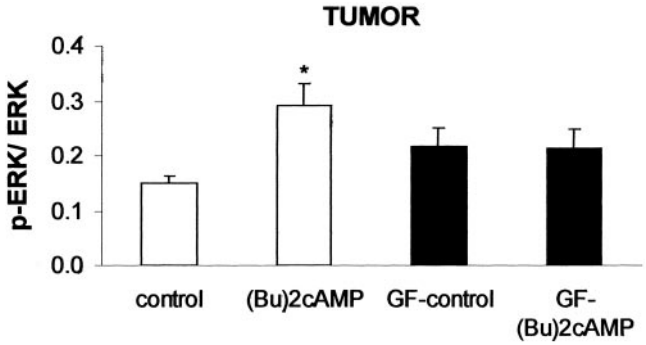

FIG. 11. Dibutiryl-cAMP [$(Bu)_2$cAMP, 1 mM]-induced ERK1/2 activation in tumor cells in the absence or presence of the PKC inhibitor GF 109203X (25 μM). Experiments were repeated three times. *, Significantly different from control in cells without pretreatments.

kinases (27) and phosphotyrosine phosphatases (11, 16). Different cells may express one or more of these pathways and the resulting complex cross-talk between the messenger molecules conditions the response to GnRH. In addition, changes in receptor number may result in differential coupling to G proteins, enabling GnRH to induce a variety of physiological end points (32). The purpose of the present work was to identify the signaling pathway/s activated by GnRH in ovarian tumor cells derived from rat-luteinized intrasplenic ovarian tumors (luteoma), in which GnRH does not stimulate the classical PLC transduction pathway (6). A lack of GnRH-induced PLC activation was also described in several other tumors (11, 17).

The determination of the expression of G_s, $G_{q/11}$, G_i, and G_0, described to couple to the GnRH receptor (32) in both tumor and control luteal cells (SPO), discarded that the atypical response regarding PLC in tumors was the consequence of an altered G protein expression.

Regarding the effect of buserelin on PLA_2 activation, in contrast to SPO cells, in tumor cells no variation in ^{3}H-AA was observed under the GnRH analog treatment. In addition, basal ^{3}H-AA acid levels were not modified by quinacrine either, suggesting that ^{3}H-AA in the culture media may not derive from basal PLA_2 action on membrane phospholipids in tumor cells. Rat ovarian PLA_2 has been shown to be calcium dependent (46, 47). In addition, in immortalized hypothalamic neurons it was demonstrated that calcium-dependent PLA_2 acts downstream of PLC (48). Therefore, the deficiency in buserelin-induced PLA_2 stimulation in tumor cells may be the consequence of lack of significant PLC ac-

tivation described in response to buserelin in these cells (6). Alternatively, it may be the consequence of a deficit of a functional PLA_2 because the positive control, PGF2α, was also devoid of effect.

In the case of PLD, basal levels of ^{3}H-PE were significantly lower in tumor than SPO cells, and the response to PMA, a known activator of this enzyme, was also considerably diminished in tumor cells. These results suggest that either PLD levels may be decreased in this cell type or the activity of the enzyme may be affected. In SPO cells the effect of buserelin on ^{3}H-PE accumulation was concentration dependent and reached saturation at 10 ng/ml, similar to what had been previously reported in granulosa cells (38). In contrast, in tumor cells only the highest concentration of the GnRH agonist was able to induce a significant response and to a lesser degree than in SPO cells. The participation of the PLC-PKC pathway in PLD activation by GnRH remains controversial and depends on the cell type studied (38, 49). In addition, a direct G protein-dependent activation of PLD by GnRH has been also proposed (38). Though buserelin action on PLD activation in our tumor cells is rather weak, the PA released may either exert direct effects on steroidogenesis (38, 50) or affect other signaling systems (*e.g.* PKC) by interconverting into DAG (21). Taken together the results on PLA_2 and PLD with the previous ones on PLC (6) suggest that phospholipases are not the main signal transduction pathways mediating GnRH effects in luteoma cells.

In a pioneer work, Borgeat *et al.* (51) demonstrated that GnRH stimulated cAMP accumulation in *in vitro* pituitary glands. The coupling of the GnRH receptor to the Gαs protein was later confirmed in gonadotrophs (34) and pituitary GH_3 (32, 52) and COS-7 (53) cells transfected with the GnRH receptor. Controversial results were reported regarding this issue in ovarian cells, with some authors reporting GnRH-induced cAMP increases (54, 55), but others reporting no effect (29, 56–58). It has also been proposed that GnRH inhibits LH or FSH-stimulated cAMP production both in immature granulosa cells and differentiated granulosa or luteal cells (54, 56, 57, 59). We observed that in both luteoma and SPO cells, buserelin significantly stimulated cAMP production. Furthermore, buserelin inhibited hCG-stimulated cAMP production only in tumor cells, parallel to our results on progesterone (19). The lack of effect of GnRH on hCG-induced cAMP production in SPO cells, although in agreement with our progesterone results, differs from the results of others (1, 56) and may be the consequence of different experimental conditions. The effect of GnRH on cAMP production was demonstrated to be $G_{\alpha i/0}$ independent.

Interestingly, PTX treatment on its own increased cAMP levels only in tumor cells, suggesting that these cells possess a basally active $G_{\alpha i/0}$ protein that may be inhibiting adenylyl cyclase function and could explain the significantly lower cAMP levels observed in tumor cells, compared with SPO cells. GnRH stimulation of the cAMP signaling pathway, although at the same time without effect on the PLC pathway, may be the consequence of GnRH receptor number expressed on tumor cells because it has been described that low receptor numbers do not interfere with cAMP production and affect IP formation (27, 36). We have previously demonstrated that GnRH receptors are 5 times less abundant in these ovarian tumors than in anterior pituitaries (19). Nevertheless, we also demonstrated that their number is significantly higher in these tumors than in SPO ovaries, in which buserelin does activate PLC (6), suggesting that although receptor number does not in itself explain the lack of PLC response in tumor cells, it may justify the GnRH-induced cAMP response in both cell types. It has been demonstrated by several methodologies that the GnRH activation of the PLC and cAMP pathways are independent (53, 60, 61), making different responses in both cell kinds possible.

Buserelin action on cAMP in tumor cells was parallel to its effects on progesterone secretion. The stimulatory action seems to respond to direct adenylyl cyclase stimulation by coupling to Gs, as has been proposed previously (34). Here we demonstrate that the inhibitory action of buserelin on hCG-induced cAMP or progesterone production is independent of $G_{i/0}$ participation and probably involves PKC action. The participation of PKC in this effect had been previously proposed by Amsterdam *et al.* (50). Moreover, it has also been described that GnRH-stimulated MAPK activity contributes to GnRH-induced progesterone inhibition in human granulosa-lutein cells (28). In preliminary experiments, we found that preincubation with the MEK1 inhibitor PD 98089 inhibited the buserelin-induced inhibition of hCG-induced progesterone secretion, in agreement with this observation, suggesting a PKC-MAPK pathway in this effect (see below). With regard to buserelin-induced PKC activation in tumor cells, it does not seem to be dependent on PLC; nevertheless, DAG originating from PLD activation could be behind the PKC activation (50). In addition, an action of PKA, stimulated by increased cAMP levels, on PKC has also been proposed (62, 63) (see below).

Because the activation of ERK1/2 had been proposed to participate in GnRH signaling in pituitary (24–27, 64) as well as ovarian cells (15, 28, 29), next we studied the effect of buserelin on ERK1/2 phosphorylation in our system. Buserelin induced significant ERK1/2 phosphorylation in both cells kinds, although the degree of stimulation was lower in tumor cells. To our knowledge, this is the first description of GnRH-induced ERK1/2 activation in rat ovarian luteinized cells. It has been previously demonstrated that the GnRH receptor can couple to $G_{i/0}$ (32), and the participation of this G protein in MAPK activation has remained controversial (24, 25). Buserelin-induced ERK1/2 activation proved to be $G_{i/0}$ independent in both cell kinds. In addition, PKC-dependent and -independent pathways had been proposed in GnRH-induced MAPK phosphorylation (26). ERK1/2 activation in tumor and SPO cells was markedly PKC dependent because PKC inhibition or down-regulation abolished the effects of buserelin or PMA in both cell types, in agreement with previous reports in pituitary (24, 25, 50) and ovarian cells (29).

Because buserelin stimulated cAMP production and this nucleotide had been involved in MAPK activation (25–27, 29), we tested whether the cAMP pathway was involved in MAPK activation by buserelin in these cells. Interestingly, the adenylyl cyclase inhibitor, SQ 22536, prevented buserelin-induced ERK1/2 phosphorylation in tumor but was without effect in SPO cells. In SPO cells, which responded to buserelin stimulation with PLC activation and increases in Ca^{2+} and IP_3 (6), ERK1/2 phosphorylation was probably due mainly

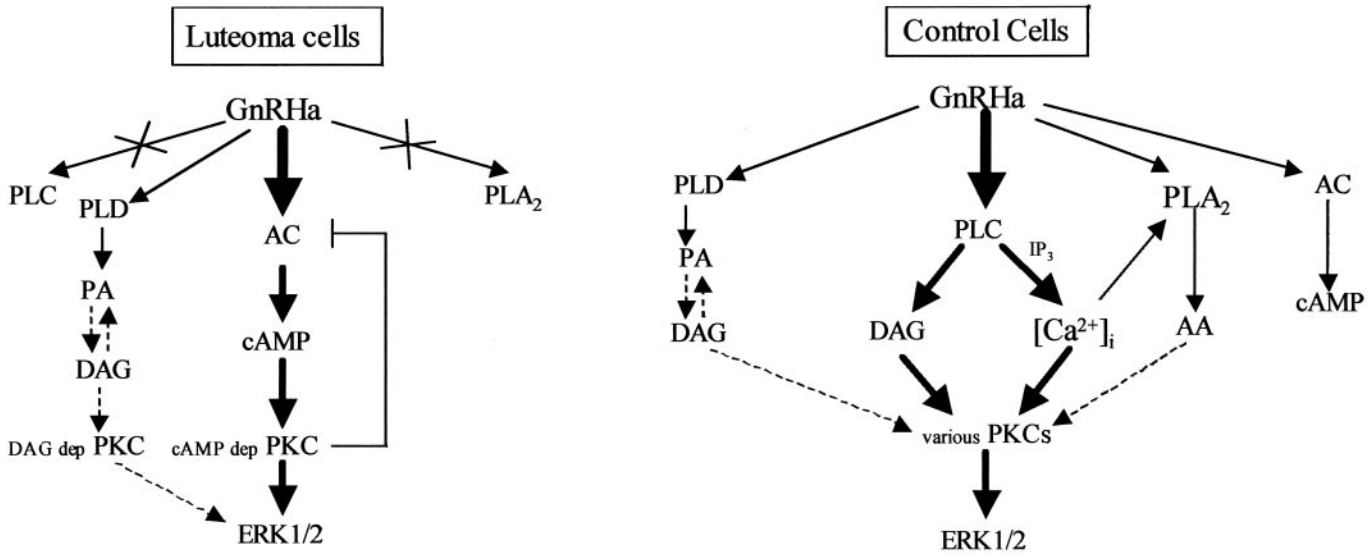

FIG. 12. Diagram proposing different GnRH signaling pathways in luteoma and normal luteal cells. GnRHa, GnRH analog; IP_3, inositol triphosphate; $[Ca^{2+}]_I$, intracellular calcium concentrations; AA, arachidonic acid; AC, adenylyl cyclase. ➡, Activation; →, weak activation; ⊣, inhibition; ⤫, nonactivated pathways.

to calcium-dependent PKC stimulation. In contrast, in tumor cells, in which buserelin does not activate PLC and marginally stimulates PLD, ERK1/2 phosphorylation seemed to be cAMP dependent, confirmed by the ERK1/2 response to dibutyryl cAMP in the presence or absence of PKC inhibitor GF109203X. Therefore, a signaling pathway including sequential activation of adenylyl cyclase-PKC seems to mediate buserelin-induced ERK1/2 phosphorylation in tumor cells. The cAMP- or PKA-induced increases in PKC activity have also been suggested in other cell types (62, 63, 65). A proposed model for GnRH signaling in luteoma *vs.* control luteal cells is shown in Fig. 12.

Taken together these results show that although phospholipases are marginally (PLD) or not at all (PLA2 and PLC) stimulated by GnRH analogs in luteoma cells, adenylyl cyclase, PKC, and ERK1/2 signal transduction pathways mediate GnRH effects in these cells. In consequence, although the transformation of the ovary into a luteoma, a benign hyperplastic tumor, implies the loss of classical GnRH signaling pathways (PLC), similar to what is observed in various malignancies (11, 17), the second messenger-generating systems determined in response to buserelin in these cells do not resemble those observed in transformed tissue either ($G_{i/0}$-activated MAPK, activation phosphotyrosine phosphatases) (12, 15–17, 35, 66). This demonstrates that GnRH signaling varies considerably in different cells and makes possible a diversity of responses depending on the pathways that are activated in each situation.

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References

1. **Clayton RN, Harwood JP, Catt KJ** 1979 Gonadotropin-releasing hormone analogue binds to luteal cells and inhibits progesterone production. Nature 282:90–92
2. **Pieper DR, Richards JS, Marshall JC** 1981 Ovarian gonadotropin-releasing hormone (GnRH) receptors: characterization, distribution, and induction by GnRH. Endocrinology 108:1148–1155
3. **Aten RF, Polan ML, Bayless R, Behrman HR** 1987 A gonadotropin-releasing hormone (GnRH)-like protein in human ovaries: similarity to the GnRH-like ovarian protein of the rat. J Clin Endocrinol Metab 64:1288–1293
4. **Peng C, Fan NC, Ligier M, Väänänen J, Leung PCK** 1994 Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. Endocrinology 135:1740–1746
5. **Olofsson JI, Conti CC, Leung PCK** 1995 Homologous and heterologous regulation of gonadotropin-releasing hormone receptor gene expression in preovulatory rat granulosa cells. Endocrinology 136:974–980
6. **Chamson-Reig A, Pignataro OP, Libertun C, Lux-Lantos V** 1999 Alterations in intracellular messengers mobilized by gonadotropin-releasing hormone in an experimental ovarian tumor. Endocrinology 140:3573–3580
7. **Gründker C, Gündhert AR, Westphalen S, Emons G** 2002 Biology of the gonadotropin-releasing hormone system in gynecological cancers. Eur J Endocrinol 146:1–14
8. **Schally AV, Comaru-Schally AM, Nagy A, Kovacs M, Szepeshazi K, Plonowski A, Varga JL, Halmos G** 2001 Hypothalamic hormones and cancer. Front Neuroendocrinol 22:248–291
9. **Leung PCK, Steele GL** 1992 Intracellular signaling in the gonads. Endocr Rev 13:476–498
10. **Cheng KW, Leung PCK** 2000 The expression, regulation and signal transduction pathways of the mammalian gonadotropin-releasing hormone receptor. Can J Physiol Pharmacol 78:1029–1052
11. **Emons G, Müller V, Ortmann O, Schultz KD** 1998 Effects of LHRH-analogs on mitogenic signal transduction in cancer cells. J Steroid Biochem Mol Biol 65:199–206
12. **Limonta P, Moretti RM, Montagnani Marelli M, Dondi D, Parenti M, Motta M** 1999 The luteinizing hormone-releasing hormone receptor in human prostate cancer cells: messenger ribonucleic acid expression, molecular size, and signal transduction pathway. Endocrinology 140:5250–5256
13. **Nicholson RI, Turkes WA, Dyas L, Plowman PN, Williams M, Blamey RW** 1985 Endocrinological and clinical aspects of LHRH action (ICI 118630) in hormone dependent breast cancer. J Steroid Biochem Mol Biol 23:834–847
14. **Kang SK, Choi K, Cheng KW, Nathwani PS, Auersperg N, Leung PCK** 2000 Role of gonadotropin-releasing hormone as an autocrine growth factor in human ovarian surface epithelium. Endocrinology 141:72–80
15. **Kimura A, Ohmichi M, Kurachi H, Ikegami H, Hayakawa J, Tasaka K, Kanda Y, Nishio Y, Jikihara H, Matsuura N, Murata Y** 1999 Role of mitogen-activated protein kinase/extracellular signal-regulated kinase cascade in gonadotropin-releasing hormone-induced growth inhibition of a human ovarian cancer cell line. Cancer Res 59:5133–5142

16. **Gründker C, Völker P, Emons G** 2001 Antiproliferative signaling of luteinizing hormone-releasing hormone in human endometrial and ovarian cancer cells through G protein αI-mediated activation of phosphotyrosine phosphatase. Endocrinology 142:2369–2380
17. **Emons G, Müller V, Ortmann O, Trautner U, von Stuckrad B, Schultz KD, Schally AV** 1996 Luteinizing hormone-releasing hormone agonist triptorelin antagonizes signal transduction and mitogenic activity of epidermal growth factor in human ovarian and endometrial cancer cell lines. Int J Oncol 9:1129–1137
18. **Lux-Lantos VAR, Thyssen SM, Chamson-Reig A, Libertun C** 1995 Effect of a gonadotropin releasing hormone analog on an experimental ovarian tumor: direct and indirect actions. Life Sci 57:291–300
19. **Chamson-Reig A, Lux-Lantos VAR, Tesone M, Libertun C** 1997 GnRH receptors and GnRH endocrine effects on luteoma cells. Endocrine 6:165–171
20. **Stojilkovic SS, Reinhart J, Catt KJ** 1994 Gonadotropin-releasing hormone receptors: structure and signal transduction pathways. Endocr Rev 15:462–499
21. **Shacham S, Cheifetz MN, Lewy H, Ashkenazi IE, Becker OM, Seger R, Naor Z** 1999 Mechanism of GnRH receptor signalling: from the membrane to the nucleus. Ann Endocrinol (Paris) 60:79–88
22. **Naor Z, Outhiriaradjou B, Seger R** 2000 Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. Trends Endocrinol Metab 11:91–99
23. **Haisenleder DJ, Cox ME, Parsons SJ, Marshall JC** 1998 Gonadotropin-releasing hormone pulses are required to maintain activation of mitogen-activated protein kinase: role in stimulation of gonadotrope gene expression. Endocrinology 139:3104–3111
24. **Sim PJ, Wolbers WB, Mitchell R** 1995 Activation of MAP kinase by the LHRH receptor through a dual mechanism involving protein kinase C and a pertussis toxin-sensitive G protein. Mol Cell Endocrinol 112:257–263
25. **Reiss N, Llevi LN, Shacham S, Harris D, Seger R, Naor Z** 1997 Mechanism of mitogen-activated protein kinase activation by gonadotropin-releasing hormone in the pituitary αT3–1 cell line: differential roles of calcium and protein kinase C. Endocrinology 138:1673–1682
26. **Han X-B, Conn PM** 1999 The role of protein kinases A and C pathways in the regulation of mitogen-activated protein kinase activation in response to gonadotropin-releasing hormone receptor activation. Endocrinology 140:2241–2251
27. **Johnson MS, Lutz EM, MacKenzie CJ, Wolbers WB, Robertson DN, Holland PJ, Mitchell R** 2000 Gonadotropin-releasing hormone receptor activation of extracellular signal-regulated kinase and tyrosine kinases in transfected GH3 cells and in αT3–1 cells. Endocrinology 141:3087–3097
28. **Kang SK, Tai C-J, Cheng KW, Leung PCK** 2000 Gonadotropin-releasing hormone activates mitogen-activated protein kinase in human ovarian and placental cells. Mol Cell Endocrinol 170:143–151
29. **Kang SK, Tai C-J, Nathwani PS, Choi K-C, Leung PCK** 2002 Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone in human granulosa-luteal cells. Endocrinology 142:671–679
30. **Kananen K, Rilianawati, Paukku T, Markkula M, Rainio E-M, Huhtamiemi I** 1997 Suppression of gonadotropins inhibits gonadal tumorigenesis in mice transgenic for the mouse inhibin α-subunit promoter/Simian virus 40 T-antigen fusion gene. Endocrinology 138:3521–3531
31. **Pierantoni R, Cobellis G, Meccariello R, Fasano S** 2002 Evolutionary aspects of cellular communication in the vertebrate hypothalamo-hypophysio-gonadal axis. Int Rev Cytol 218:69–141
32. **Stanislaus D, Pinter JH, Janovick JA, Conn PM** 1998 Mechanisms mediating multiple physiological responses to gonadotropin-releasing hormone. Mol Cell Endocrinol 144:1–10
33. **Stanislaus D, Janovick JA, Brothers S, Conn PM** 1997 Regulation of Gq/11α by the gonadotropin-releasing hormone receptor. Mol Endocrinol 11:738–746
34. **Stanislaus D, Ponder S, Ji TH, Conn PM** 1998 Gonadotropin-releasing hormone receptor couples to multiple G proteins in rat gonadotrophs and in GGH3 cells: evidence from palmitoylation and overexpression of G proteins. Biol Reprod 59:579–586
35. **Imai A, Takagi H, Horibe S, Fuseya T, Tamaya T** 1996 Couplin of gonadotropin-releasing hormone receptor to Gi protein in human reproductive tract tumors. J Clin Endocrinol Metab 81:3249–3253
36. **Pinter JH, Janovick JA, Conn PM** 1999 Gonadotropin-releasing hormone receptor concentration differentially regulates intracellular signaling pathways in GGH3 cells. Pituitary 2:181–190
37. **Lux VAR, Tesone M, Larrea GA, Libertun C** 1984 High correlation between prolactinemia, 125-I hLH binding and progesterone secretion by an experimental luteoma. Life Sci 35:2345–2352
38. **Liscovitch M, Amsterdam A** 1989 Gonadotropin-releasing hormone activates phospholipase D in ovarian granulosa cells. J Biol Chem 264:11762–11767
39. **Watanabe H, Tanaka S, Akino T, Hasegawa-Sasaki H** 1990 Evidence for coupling of different receptors for gonadotropin-releasing hormone to phospholipases C and A2 in cultured rat luteal cells. Biochem Biophys Res Commun 168:328–334
40. **Omata K, Abraham N, Schwartzman M** 1992 Renal cytochrome P-450-arachidonic acid metabolism: localization and hormonal regulation in SHR. Am J Physiol 262:F591–F599
41. **Del Punta K, Charreau E, Pignataro OP** 1996 Nitric oxide inhibits Leydig cell steroidogenesis. Endocrinology 137:5337–5343
42. **Naor Z, Catt KJ** 1981 Mechanism of action of gonadotropin-releasing hormone: involvement of phospholipid turnover in luteinizing hormone release. J Biol Chem 256:2226–2229
43. **Netiv E, Liscovitch M, Naor Z** 1991 Delayed activation of phospholipase D by gonadotropin-releasing hormone in a clonal pituitary gonadotrope cell line (αT3–1). FEBS Lett 295:107–109
44. **Minegishi T, Leung PCK** 1985 Luteinizing hormone-releasing hormone stimulates arachidonic acid release in rat granulosa cells. Endocrinology 117:2001–2007
45. **Kraus S, Naor Z, Seger R** 2001 Intracellular signaling pathways by the gonadotropin-releasing hormone (GnRH) receptor. Arch Med Res 32:499–509
46. **Minegishi T, Wang J, Leung PCK** 1987 Luteinizing hormone-releasing hormone (LHRH)-induced arachidonic acid release in rat granulosa cells. Role of calcium and protein kinase C. FEBS Lett 214:139–142
47. **Kol S, Ruutiainen-Altman K, Ben-Shlomos I, Payne DW, Ando M, Adashi EY** 1997 The rat ovarian phospholipase A_2 system: gene expression, cellular localization, activity characterization, and interleukin-1 dependence. Endocrinology 138:322–331
48. **Kreda SM, Sumner M, Fillo S, Ribeiro CM, Luo GX, Xie W, Daniel KW, Shears S, Collins S, Wetsel WC** 2001 α_1-Adrenergic receptors mediate LH-releasing hormone secretion through phospholipases C and A_2 in immortalized hypothalamic neurons. Endocrinology 142:4839–4851
49. **Zheng L, Stojilkovic SS, Hunyady L, Krsmanovic LZ, Catt KJ** 1994 Sequential activation of phospholipase C and phospholipase D in agonist-stimulated gonadotrophs. Endocrinology 134:1446–1454
50. **Amsterdam A, Dantes A, Liscovitch M** 1994 Role of phospholipase-D and phosphatidic acid in mediating gonadotropin-releasing hormone-induced inhibition of preantral granulosa cell differentiation. Endocrinology 135:1205–1211
51. **Borgeat P, Chavancy G, Dupont A, Labrie F, Arimura A, Schally A** 1972 Stimulation of adenosine 3′, 5′-cyclic monophosphate accumulation in anterior pituitary gland *in vitro* by synthetic luteinizing hormone-releasing hormone. Proc Natl Acad Sci USA 69:2677–2681
52. **Kuphal D, Janovick JA, Kaiser UB, Chin WW, Conn PM** 1994 Stable transfection of GH3 cells with rat gonadotropin-releasing hormone receptor complementary deoxyribonucleic acid results in expression of a receptor coupled to cyclic adenosine 3′, 5′-monophosphate-dependent prolactin release via a G-protein. Endocrinology 135:315–320
53. **Arora KK, Krsmanovic LZ, Mores N, O'Farrell H, Catt K** 1998 Mediation of cyclic AMP signaling by the first intracellular loop of the gonadotropin-releasing hormone receptor. J Biol Chem 273:25581–25586
54. **Ranta T, Knecht M, Kody M, Catt KJ** 1982 GnRH receptors in cultured rat granulosa cells: mediation of the inhibitory and stimulatory actions of GnRH. Mol Cell Endocrinol 27:233–240
55. **Knecht M, Amsterdam A, Catt KJ** 1981 The regulatory role of cyclic AMP in hormone-induced of granulosa cell differentiation. J Biol Chem 256:10628–10633
56. **Behrman HR, Preston SL, Hall AK** 1980 Cellular mechanism of the anti-gonadotropic action of luteinizing hormone-releasing hormone in the corpus luteum. Endocrinology 107:656–664
57. **Massicotte J, Borgus JP, Lachance R, Labrie F** 1981 Inhibition of hCG-induced cyclic AMP accumulation and steroidogenesis in rat luteal cells by an LHRH agonist. J Steroid Biochem 14:239–242
58. **Eckstein N, Eshel A, Eli Y, Ayalon D, Naor Z** 1986 Calcium-dependent actions of gonadotropin-releasing hormone agonist and luteinizing hormone upon cyclic AMP and progesterone production in rat ovarian granulosa cells. Mol Cell Endocrinol 47:91–98
59. **Harwood JP, Clayton RN, Catt KJ** 1980 Ovarian gonadotropin-releasing hormone receptors. I. Properties and inhibition of luteal cell function. Endocrinology 107:407–413
60. **Awara WM, Guo C-H, Conn PM** 1996 Effects of Asn^{318} and $Asp^{87}Asn^{318}$ mutations on signal transduction by the gonadotropin-releasing hormone receptor and receptor regulation. Endocrinology 137:655–662
61. **Lin X, Janovick JA, Conklin D** 1998 Mutations at the consensus phosphorylation sites in the third intracellular loop of the rat gonadotropin-releasing hormone receptor: effect on receptor ligand binding and signal transduction. Biol Reprod 59:1470–1476
62. **Rosado E, Schwartz Z, Sylvia VL, Dean DD, Boyan BD** 2002 Transforming growth factor-beta 1 regulation of growth zone chondrocytes is mediated by multiple interacting pathways. Biochim Biophys Acta 1590:1–15
63. **Yu PY, Eisner GM, Yamaguchi I, Mouradian MM, Felder RA, Jose PA** 1996 Dopamine D1A receptor regulation of phospholipase C isoform. J Biol Chem 271:19506–19508
64. **Sundaresan S, Colin IM, Pestell RG, Jameson JL** 1996 Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone: evidence for the involvement of protein kinase C. Endocrinology 137:304–311
65. **Takekoshi K, Ishii K, Nanmoku T, Shibuya S, Kawakami Y, Isobe K, Nakai T** 2001 Leptin stimulates catecholamine synthesis in a PKC-dependent manner in cultured porcine adrenal medullary chromaffin cells. Endocrinology 142: 4861–4871
66. **Gründker C, Schlotawa L, Viereck V, Emons G** 2001 Protein kinase C-independent stimulation of activator protein-1 and c-Jun N-terminal kinase activity in human endometrial cancer cells by the LHRH agonist triptorelin. Eur J Endocrinol 145:651–658