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Concerted Action of Human Chorionic Gonadotropin and Norepinephrine on Intracellular-Free Calcium in Human Granulosa-Lutein Cells: Evidence for the Presence of a Functional α-Adrenergic Receptor*

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ABSTRACT

Luteal cells are known to possess receptors for LH/hCG and receptors of the β-adrenergic type. Interactions of specific agonists with either receptor lead to the activation of adenylate cyclase and subsequently to an increase of cAMP. Since in the human there is also evidence for the presence of α-adrenergic receptors, we have investigated whether activation of these receptors is linked to calcium as a second messenger and performed measurements of intracellular free calcium (Ca^{2+}) with Fura-2 in single human granulosa-lutein cells. Addition of either hCG (100, 1,000, 25,000 IU/L) or norepinephrine (NE; known to interact with both α- and β-adrenergic receptors), β-adrenergic receptor agonist isoproterenol (ISO), or α-adrenergic receptor agonist phenylephrine (PHE; all at 10 and 100 μmol/L) did not increase free intracellular Ca^{2+}. However, the addition of combinations of NE/hCG, PHE/hCG, but not the combination ISO/hCG, induced a transient increase in cytosolic free Ca^{2+}. The NE/hCG-evoked calcium signal was not abolished in the presence of the β-adrenergic receptor antagonist propranolol and was not affected by removal of extracellular Ca^{2+}. Furthermore, we tested whether catecholamines affected the release of progesterone in the presence or absence of hCG. As expected, hCG (10,000 IU/L) stimulated progesterone release by cultured granulosa-lutein cells. When these cells were incubated with NE, PHE, or ISO (at 10 μmol/L), production of progesterone by these cells was not affected. However, the combinations of NE and PHE with hCG abolished the hCG-induced progesterone accumulation, but ISO coincubated with hCG did not. Taken together, our results indicate: 1) the presence of functional α-adrenergic receptors on human granulosa-lutein cells; 2) simultaneous activation of two different receptors (for hCG and α-agonists) are able to evoke intracellular Ca^{2+} elevation, implicating postreceptor interactions in human granulosa lutein cells; 3) this process occurs even in the absence of extracellular Ca^{2+}, indicating the involvement of intracellular Ca^{2+} stores, most likely due to activation of phosphoinositide pathway; 4) catecholamines most likely acting via α-adrenergic receptors, inhibit the LH/hCG-induced release of progesterone. (*J Clin Endocrinol Metab* **76:** 367–373, 1993)

THERE IS NO doubt that the primate corpus luteum requires the luteotrophic support of LH or of hCG, respectively. Both hormones bind to the same receptor present on granulosa and granulosa-lutein cells (1, 2), which is coupled to adenylate cyclase. cAMP stimulates the production of progesterone by granulosa lutein cells by altering cholesterol metabolism and activating the activities of steroidogenic enzymes (*cf.* 1).

Beside regulation by gonadotropins, modulatory influence of the ovarian innervation (3) on the function of the corpus luteum has been proposed (review in Ref. 4). Thus, a large body of evidence indicates that catecholamines stimulate the progesterone production by the ovary *in vivo* and by granulosa/granulosa-lutein cells *in vitro* in a variety of species, including the rat (5, 6), the sheep (7), the cow (8, 9) and the cat (10). Recently it has been reported that both epinephrine and norepinephrine (NE) stimulate progesterone secretion by human cultured granulosa cells most likely via interaction with the β-adrenergic receptor (11). Interestingly, however, the evidence for a functional role of β-adrenergic receptors in the human corpus luteum is not unequivocal and there is some evidence for the presence of an α-adrenergic receptor type in the human corpus luteum (12).

The signal transduction mechanisms involved in the release of luteal cell-derived relaxin and of progesterone as well, appear not to be coupled solely to cAMP, but also to be stimulated by the inositol phospholipid pathway (2, 13). Thus, protein kinase C activation by phorbol esters resulted in the release of relaxin by porcine luteal cells in culture (14) and maitotoxin, an activator of calcium channels, increased relaxin secretion by porcine luteal tissue without altering cAMP (15). The production of progesterone by isolated bovine luteal cells was stimulated by phorbol esters (16), whereas elevation of intracellular Ca^{2+} in ovine large luteal cells (*e.g.* by the luteolytic agent prostaglandin F 2-α) inhibited progesterone secretion (17). The Ca^{2+} ionophore A23187 alone did not affect progesterone synthesis by bovine corpora lutea but enhanced the ability of LH to increase progesterone synthesis in cells of midcycle (18). Thus, although protein

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kinase C is present in ovarian granulosa and luteal cells (*cf.* 19), its precise functional involvement remains to be established.

Recently it has been reported that FSH, which in its target cells (granulosa and Sertoli cells) is able to stimulate the production of cAMP (20, 21), also increases intracellular-free Ca^{2+} levels in swine granulosa cells (22). We have therefore suspected that besides cAMP, an increase in cytosolic-free Ca^{2+} concentration could be evoked by the major hormonal stimulus, hCG, and by nerval influences mediated by (α-adrenergic) catecholamines as well, and have examined this possibility in cultured human granulosa-lutein cells.

Subjects and Methods

Materials

Fura-2 and Fura2/AM (pentaacetoxymethyl ester) were purchased from Calbiochem (La Jolla, CA), ethyleneglycol-bis (β-aminoethyl ether)-*N-N'*-tetraacetic acid (EGTA) was bought from Fluka (Neu Ulm, Germany), medium DME-F12 from Sigma (Munich, Germany), and fetal calf serum from GIBCO (Berlin, Germany). Human CG, (−)norepinephrine (NE), (L)isoproterenol, (L)phenylephrine, (D/L)propranolol, and all other chemicals were from Sigma.

Methods

Culture of human granulosa-lutein cells. The procedure was described previously in detail (23). In brief, human granulosa cells were isolated from follicular fluid obtained from a total of 16 women in connection with the *in vitro* fertilization program at the Universitätsfrauenklinik Ulm. The patients were treated with standard hormonal regimes prior to follicular puncture (stimulation with human menopausal gonadotropin, monitoring of follicular growth by ultrasound, and daily estradiol, progesterone, and LH measurement; 24). Ovulation was induced by 10,000 IU hCG as the follicles reached a diameter of 17 mm. Follicular aspiration was performed 32–36 h after induction of ovulation under sonographic control in all women with a continuous rise in serum estradiol.

For the calcium measurements in the present study, we have twice used pools of aspiration fluids of two patients, as well as not pooled follicular fluid of three other patients (total of 7 women). For the measurements of progesterone secretion, cells from additional nine women were used (three pools of three women each). In all cases, cells were mechanically dispersed by repeated aspiration into a 5-mL pipette and through a 20-g needle, fluid was then mixed with dextran T 250 (4.5%; 5:1; Roth, Karlsruhe, Germany) and placed into the incubator for 30 min. The supernatant was removed and centrifuged (approximately 1000 × *g*, 3 min). The pellets containing granulosa cells were washed with Ham's F12 (1:1) and plated with Dulbecco's modified Eagle's medium:Ham's F12 (1:1) with 10% fetal calf serum in 60-mm Falcon (Becton-Dickinson, Heidelberg) culture dishes. Cells were kept in an incubator at a humidified atmosphere with 5% CO_2 at 37 C. After 24 h media was replaced and all nonadherent cells (mainly red blood cells) were washed off. Subsequently, medium was changed every 2 days, and cells were used for the calcium measurements on the third or fourth day after plating.

Fura-2 loading

The cells were loaded with Fura-2/AM in the culture medium without serum (15–30 min at 37 C). Fura-2 acetoxymethyl ester (Fura-2/AM, dissolved in dimethyl sulfoxide) was added from a stock to give, finally, 1.5 μmol/L Fura-2/AM and 0.1% dimethyl sulfoxide. Subsequently, the cells were washed with solution A (in mmol/L: 140 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1 $CaCl_2$, 0.5 ascorbic acid, 11 glucose, 15 1,4-piperazinediethanesulfonic acid, pH 7.2) and used for Ca^{2+} measurements 1–4 h after dye loading. Solution A was modified by adding 1 mmol/L EGTA instead of $CaCl_2$ to perform measurements in the absence of extracellular Ca^{2+}.

Microscopic fluorescence measurements of cell cluster and single cells

Fluorescence measurements were performed with the Zeiss Microscope Photometer System as described previously (23). Fluorescence light from an area adjusted to the cell size was collected (usually 400 μm^2 for single cell observation or approximately 2500 μm^2 for cell clusters of 3–5 cells). The autofluorescence of unloaded cells was not taken into account since it was less than 1% of the fluorescence of dye loaded cells. Background was measured in a cell-free area which contributed to about 5–10% of the signal obtained from Fura-2 loaded cells. This value and the value of the dark current from the photomultiplier (noise of the photomultiplier in the complete dark, depending on the high-voltage adjustment) were subtracted from cell signals. With these corrected values, ratio calculations of the 340-nm recordings divided by the 380-nm recordings were carried out according to Grynkiewicz *et al.* (25).

Calibration of the Fura-2 signal

The Fura-2 signals were calibrated with solution B (150 mmol/L KCl, 20 mmol/L 3-(*N*-morpholino)propanesulfonic acid, pH 7.2) which was routinely used to mimic the intracellular fluid when analyzing intracellular Ca^{2+} fluxes with permeabilized endocrine cell preparations (26). Solution B, supplemented with 5 μmol/L of the free acid of Fura 2 and either 5 mmol/L Ca^{2+} or 5 mmol/L EGTA were used to determine R_{max}, R_{min}, and β. With the experimental setup, R_{max} was 8.5, R_{min} was 0.25, and β was 8.5. For the calculation of free Ca^{2+} concentrations an association constant of the Fura-2/calcium of 224 nmol/L was assumed as given by Grynkiewicz *et al.* (25).

Addition of test substances

Test substances were added with a motor driven syringe (Hamilton microlab P system, speed setting 9) connected with tubings. Its end consisted of an Eppendorf comfort tip which was mounted in a distance of approximately 3 mm from the cells in the culture dishes. All test substances were dissolved in solution A and were added in a total volume of 25 or 50 μL to 2 mL buffer in the culture plate. The consecutive treatment with several test substances was carried out by handpipetting. All concentrations reported in the following are undiluted concentration in the syringe before addition to the cells.

Measurements of progesterone release: incubation experiments

A total of three incubation experiments were performed, each with pooled cells harvested from three women. Experiments were started 18–20 h after plating and all nonadherent cell were removed by repeated washing of the plates with culture media. Cells were then exposed to fresh culture media containing ascorbate [(AA), 100 μg/mL) control groups], catecholamines [NE, phenylephrine (PHE), isoproterenol (ISO), at 10 μmol/L in media containing 100 μg/mL AA] and/or hCG (at 10,000 IU/L dissolved in media containing AA at 100 μg/mL). Total volume per dish was 3 mL. After 6 h the supernatants were collected and frozen until measurement of progesterone and estradiol using routine RIA protocols in the Universitätsfrauenklinik Ulm as described (24). Cells were harvested and protein concentration per well was determined with the help of the BCA (Pierce, Rockford, IL) method. The accumulation of progesterone was expressed as nanograms per milligram of protein. In order to compare the results of the three incubations, data are expressed as percentage of control release in this report.

Statistics

Data from progesterone measurements were evaluated by analysis of variance followed by the Scheffe F test and/or the Fisher test as specified in *Results*.

Results

Cell culture

Individual human granulosa cells were used for measurements of intracellular-free calcium after cultivation for 3 or 4 days. At this time the cells (Fig. 1) resembled the cell type described by McAllister *et al.* (27).

Ca^{2+} measurements

After Fura-2 loading the granulosa cells showed a homogeneous distribution of the dye, indicating a negligible loading of cell organelles. The basal value of intracellular-free Ca^{2+} ranged between 70 and 150 nmol/L (n = 113 individual cells, see values in Figs. 2–5 before stimulation). The values were not significantly different among various cell preparations. With the exception of one cell, the basal Ca^{2+} value was stable and no spontaneous oscillations of intracellular free Ca^{2+} were observed (not shown).

Neither 100, 1,000 and 25,000 IU hCG (25 out of 25 cells; *cf.* Fig. 2, in which addition of hCG had no stimulatory effect) nor 10–100 μmol/L ISO (11 out of 12 cells), PHE (11 out of 12 cells), or NE (11 out of 11 cells; *cf.* Fig. 3) alone evoked an increase in cytosolic free Ca^{2+}. (Note that all numbers of cells refer to individual cells in pooled and nonpooled cell preparations.) However, when NE and hCG were added one after the other within 10–30 s (see Fig. 2), irrespectively of the sequence of addition, or if NE was added together with hCG (Fig. 3) a transient increase of cytosolic-free calcium was observed within a few seconds. No difference in the signalling pattern was observed if we used naive cells or pretreated cells. The mean peak of intracellular free Ca^{2+} ranged between 600 and 800 nmol/L Ca^{2+}. After 1 min of stimulation the Ca^{2+} concentration returned either directly to values near to the baseline or a second but smaller Ca^{2+} transient was observed (*cf.* Fig. 2 with Fig. 4). When hCG/NE were added in combination, a sharp and immediate increase in cytosolic free Ca^{2+} occurred (Fig. 3). On the basis of the single cell analysis, 26 out of 31 cells tested showed this behavior and when analyzing cell clusters (usually 5 cells) all clusters reacted positively to the NE/hCG combination (Fig. 3). Removal of Ca^{2+} in the medium by EGTA did not abolish the hCG/NE effect (5 out of 8 cells tested; Fig. 5). A positive response was also found for three out of six cells with the combination PHE/hCG (Fig. 4). When we replaced the α-agonist PHE by the β-agonist ISO, 7 out of 8 cells showed no response to the combination ISO/hCG (Fig. 4; the fact that the combination ISO/hCG had reached the cell is indicated by the downward deflection of both the 340- and 380-nm excitation signals in the *upper two lines* of Fig. 4). Since the presence of the β-receptor-antagonist propranolol (100 μmol/L) in the medium did not abolish the NE/hCG-evoked calcium signal, the response of the cells can be attributed to the interaction of NE with α-adrenergic receptors. Further investigation of the specific nature of the α-adrenoreceptor using α-1-adrenergic receptor antagonist prazosin in this experimental setup was hampered due to interference of the excitation spectrum of prazosin with the wavelengths used for Fura-2 measurements (Föhr, K.J. and A. Mayerhofer, unpublished observation).

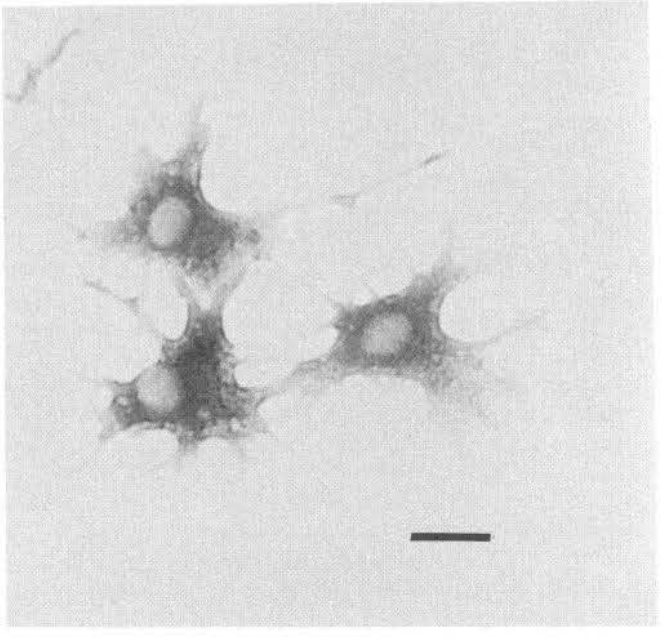

FIG. 1. Cultured human granulosa-lutein cells after 3 days of cultivation. Cells were fixed after Ca^{2+} measurements in 4% paraformaldehyde and stained with hemalaun for 5 min. *Bar*, 20 μm.

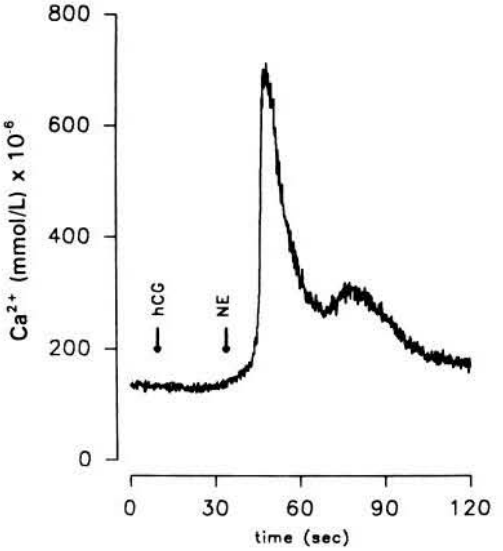

FIG. 2. Measurement of intracellular-free Ca^{2+} in single human granulosa-lutein cells using Fura-2 microfluorimetry: time course of changes in intracellular free Ca^{2+} in response to hCG and NE in single human granulosa-lutein cells. Fura-2 loading was carried out in the presence of 1.5 μmol/L Fura-2/AM for 30 min at 37 C. During the fluorescence measurements of 340 and 380 nm excitation, the cells were kept at room temperature. From the ratio R = F340/F380, the cytosolic Ca^{2+} concentrations were calculated according to Grynkiewicz *et al.* (25) assuming a K_d of 224 nmol/L. Length of recordings were generally 2-min intervals. hCG (1000 IU/L) added at the time indicated by the *arrow* did not change intracellular free Ca^{2+} levels. The addition of NE (100 μmol/L) to the same cell evokes a delayed transient elevation of Ca^{2+} followed by a smaller second transient Ca^{2+} elevation. Test substances were added in a volume of 25 μL in a distance of 3 mm from the cell to the medium (total volume in the dish was 2 mL). All concentrations give the concentration inside the pipette.

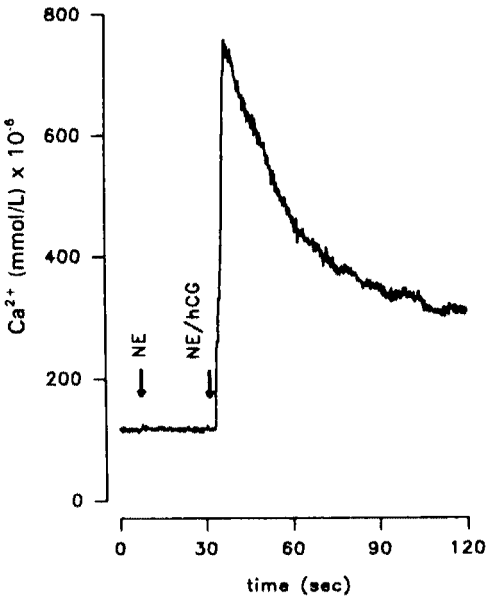

FIG. 3. Time course of changes in intracellular-free Ca^{2+} in response to NE and NE/hCG by a cluster of human granulosa-lutein cells. Experimental conditions as in Fig. 2. NE (100 μmol/L) added to the cells cluster at the time indicated by the *arrow* did not change cytosolic Ca^{2+}. The addition of the combination NE/hCG (100 μmol/L and 1000 IU/L, respectively) evoked a rapid increase of intracellular free Ca^{2+}.

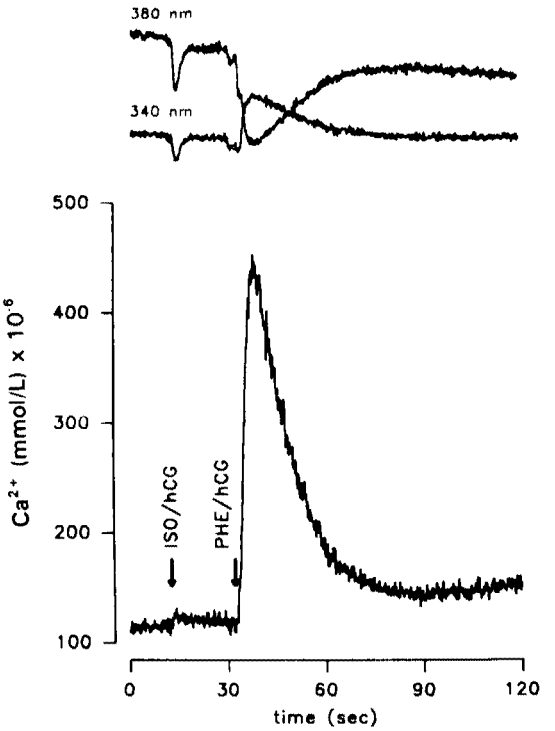

FIG. 4. Time course of changes in intracellular-free Ca^{2+} in response of ISO/hCG and PHE/hCG in single human granulosa-lutein cell. Experimental conditions as in Fig. 2. The combination of ISO/hCG (100 μmol/L, 1000 IU/L) did not increase cytosolic Ca^{2+}. PHE/hCG (100 μmol/L, 1000 IU/L) added to the same cell evoked a rapid increase in cytosolic-free Ca^{2+}. The *upper two lines* of this figures show the original recordings at 340 and 380 nm. The downward deflection of both excitation signals after the addition of ISO/hCG indicates that the drugs diluted in buffer are effectively brought to the cell, because this effect was reproducible with buffer alone. Only the simultaneous increase in the 340-nm and decrease in the 380-nm signal results in a Ca^{2+} increase of cytosolic-free Ca^{2+} after the addition of PHE/hCG.

Results of progesterone release experiments

The addition of NE, PHE, or ISO to the cell did not increase progesterone values in the culture media over basal release values, whereas hCG stimulated progesterone accumulation within 6 h. The combination of hCG with NE (Fig. 6A), as well as the combination of hCG with PHE (not shown), significantly diminished the hCG-stimulated progesterone release. In contrast, ISO did not modify the hCG-induced rise in progesterone (Fig. 6B).

Discussion

The endocrine cells of a corpus luteum are composed of two cell populations, the large granulosa-lutein cells and the small theca-lutein cells (2). In the present study we have examined granulosa-lutein cells, because we have used aspirated granulosa cells which, in culture as well as *in vivo*, undergo luteinization (*cf.* 11). Luteinization under the culture conditions of the present study is indicated by accumulation of progesterone in the culture medium of these cells (estradiol was not detectable by RIA; Mayerhofer, A. and K. Sterzik, unpublished data).

It is well established that luteal cells of a variety of species are endowed by β-adrenergic receptors (see *Introduction*). Activation of this receptor leads to an increase of cAMP in all tissues examined. In cultured human granulosa cells this mechanism appears to be responsible for the catecholamine-stimulated production of progesterone in one study (11). However, results of another study (12) do not support a stimulatory effects of β-adrenergic agonists on the progesterone production by human luteal cells. The results of this study (12) rather suggest an inhibitory effect, possibly mediated via α-adrenergic receptors on progesterone production in the presence of 17-β-estradiol. To our knowledge, however, the presence of α-receptors on granulosa-lutein cells has not been reported. Our findings that neither NE (acting on α- and β-adrenergic receptors) nor PHE (acting on α-adrenergic receptors) alone induced an increase in cytosolic free Ca^{2+}, did not allow us to draw any conclusions concerning the presence of α-adrenergic receptors. However, clear evidence for the presence of α-adrenergic receptors coupled to Ca^{2+}, is given by our data showing that the combination NE/hCG and less frequently PHE/hCG (PHE is a less potent α-adrenergic agonist than NE), but not ISO/hCG evoked an increase in cytosolic free Ca^{2+}, which was not prevented by β-adrenergic receptor antagonist propranolol. Interestingly, simultaneous presence of NE (or PHE) with hCG was required to evoke intracellular free Ca^{2+}. These results indicate not only that α-adrenergic receptors, coupled to increases of free intracellular Ca^{2+}, are present on human granulosa-lutein cells, but also that interactions of different signaling pathways take place in human granulosa-lutein cells. At present one can only speculate that such interaction are likely to occur at the level of G-proteins and/or subsequent steps. Synergistic interactions between different receptors in regu-

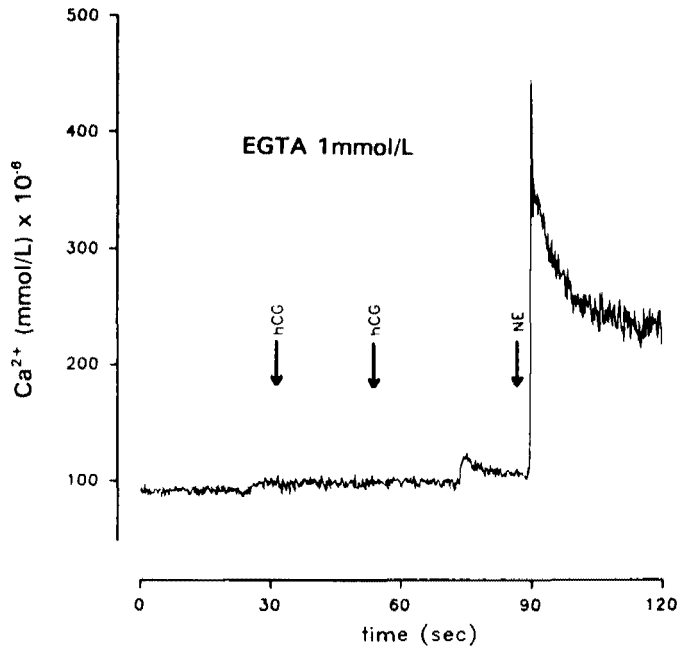

FIG. 5. Results of an experiment performed in the absence of Ca^{2+} in the media: Intracellular-free Ca^{2+} of a single human granulosa-lutein cell was not elevated in response to hCG (1000 IU/L). But subsequent addition of NE (100 μmol/L) evoked elevation of Ca^{2+}. Experimental conditions as in Fig. 2, except that 1 mmol/L EGTA was added to the media (solution A) instead of $CaCl_2$.

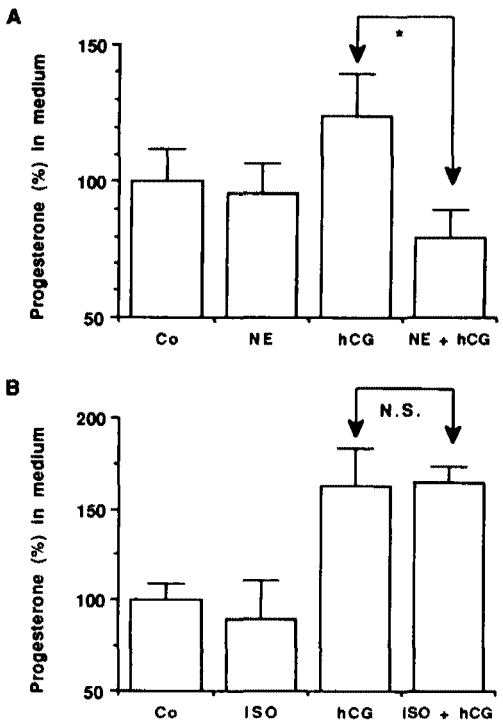

FIG. 6. A and B, Results of 6-h incubations of granulosa-lutein cells with catecholamines and hCG. The release of progesterone into the media is expressed as percentage of basal release (Co). For each experiment the pooled cells of three women were used and each treatment group represents triplicate experiments. Values are means ± SEM. Note the important statistically significant differences between incubations with hCG + NE/hCG and hCG + ISO/hCG (analysis of variance followed by Scheffe F test or Fisher test). NE, 10 μmol/L; ISO, 10 μmol/L; hCG, 10,000 IU/L.

lating signal transduction mechanisms are known to occur in a variety of tissues (*cf.* 28) including the luteal cell (*cf.* 29). However, at present, the best example for synergistic interactions between adrenergic receptors and receptors coupled to cAMP, is the pineal (*cf.* 28). Thus, the enzyme activity of N-acetyl transferase is stimulated by β-adrenergic receptor agonists, but is normally unaffected by α-adrenergic agonists. In combination, α- and β-adrenergic receptor agonists produce a much greater stimulation of this enzyme than found with β-adrenergic receptor agonist alone. α-Adrenergic agonists, while having no effect on their own, can potentiate increase of cAMP (stimulated by activation of β-adrenergic receptors) via activation of protein kincase C (PKC) and Ca^{2+} in pinealocytes (28, 30). Our results suggest similar effects in the granulosa-lutein cells. Thus, the interactive effects of catecholamines on LH/hCG regulated functions of luteal cells could be the consequence.

In general, cell activation by α-adrenergic agonists is coupled to the hydrolysis of phosphatidyl inositides which leads to the production of diacylglycerol and inositol 1,4,5-trisphosphate (28). The latter causes an increase in cytosolic-free Ca^{2+} by releasing intracellularly stored Ca^{2+}. This is very likely the case in human granulosa-lutein cells, since the effect of hCG/NE was not affected by removal of extracellular Ca^{2+} in the present study. While numerous studies show that the production of progesterone by luteal cells is stimulated via cAMP (for review see Ref. 1), it has recently been reported that the phosphoinositide pathway is present as well in luteal cells and, moreover, can lead to the secretion of progesterone and of the peptide hormone relaxin (16, 14; see introduction for further references). Relaxin is present in human large luteal cell (equivalent to granulosa-lutein cells; 31) and released by exocytosis (32). It is therefore rather tempting to speculate that Ca^{2+}, known to regulate the process of exocytosis (for review see Ref. 33), could be involved in the release of relaxin. This possibility requires further investigation.

Our data indicate inhibitory action of catecholamines on hCG-stimulated progesterone release *in vitro*. This inhibitory effect of catecholamines appeared to be mediated via α-adrenergic receptors, because it is only seen if PHE and NE were used and was not observed with ISO. It is conceivable that these results can be viewed in connection with the description of a functional, Ca^{2+}-mobilizing, α-adrenoreceptor in the present study. Additional support for this notion comes from a previous report, in which an inhibitory effect of Ca^{2+} on the activity of adenylate cyclase in human corpus luteum has been described (34). Thus, our data obtained under the conditions of concomitant LH/hCG-receptor activation (adenylate activation) and α-adrenergic receptor activation (generating intracellular free Ca^{2+}), may indicate that steps of the biosynthetic pathway of progesterone are inhibited by Ca^{2+}.

The catecholamines NE and epinephrine are present under physiological conditions in the ovary (see review in Refs. 35 and 36). Most of the NE is obviously released from the dense ovarian sympathetic innervation, implicating that local cat-

echolamine concentration can be rather high but, in addition, catecholamines can reach the ovary via bloodstream as well. Interestingly, NE concentrations vary during the cycle not only in the rat or cat ovary (*cf.* 37) but in the human corpus luteum as well, with an increase in the content of NE occurring in the midluteal phase of the human corpus luteum (37). Thus, based on our findings *in vitro*, we would like to propose that *in vivo*, in the human corpus luteum, concerted action of catecholamines and LH/hCG are likely to be involved in the fine-tuning of luteal cell function. The concept that ovarian function is not only regulated by gonadotropins, but also by its innervation has been proposed, *e.g.* for the mouse. In this species normal follicle maturation requires local release of NE before gonadotropin stimulation (38, 39; reviews in 3 and 35; see also Ref. 4). Our findings provide additional evidence for a concept of dual regulation of human ovarian function by concerted action of hormones and neurotransmitters.

In the present study, approximately 80% of the single cells analyzed (from a total of seven patients) responded in the described way to NE/hCG or PHE/hCG. Interestingly, the cells derived from one patient did not respond at all to NE/hCG. Besides interindividual differences (lack of receptors), one should keep in mind that the physiological state of the cells (including the Fura loading) cannot be judged adequately by observation only. Moreover, functional heterogeneity of human follicular cells in terms of steroid hormone production has been described (40) and granulosa cells within the follicle exhibit functional differences regarding the presence of receptors (*e.g.* LH and PRL receptors; 2). Likewise, differences in the number of α-adrenergic receptors cannot be excluded.

In summary, we have shown the usefulness of single cell Ca^{2+} measurements to examine receptor-mediated effects. With this method we have obtained evidence for the presence of functional, Ca^{2+}-mobilizing α-adrenergic receptors on human granulosa-lutein cells. The data show synergistic post-receptor interactions of different signal transduction pathways in human granulosa-lutein cells, which result in the inhibition of progesterone release by α-adrenergic catecholamines and hCG. These results let us suspect a functional role for catecholamines in the modulation of granulosa-lutein cell function in the human.

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References

1. **Hsueh AJW, Adashi EY, Jones PBC, Welsh TH**. 1984 Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev. 5:76–127.
2. **Niswender GD, Nett TM**. 1988 The corpus luteum and its control. In: Knobil E, Neill J, Ewing LL, Markert CL, Greenwald GS, Pfaff DW, eds. The Physiology of Reproduction. New York: Raven Press; 489–525.
3. **Burden, HW**. 1978 Ovarian Innervation. In: Jones RE, ed. The Vertebrate Ovary. New York: Plenum Press; 615–638.
4. **Tsafriri A**. 1988 Local nonsteroidal regulation of ovarian function. In: Knobil E, Neill J, et al., eds. The Physiology of Reproduction. New York: Raven Press; 527–565.
5. **Ratner A, Sanborn CR, Weiss GK**. 1980 Beta-adrenergic stimulation of cAMP and progesterone in rat ovarian tissue. Am J Physiol. 239:E139–E143.
6. **Norjavaara E, Selstam G, and Ahren K**. 1982 Catecholamine stimulation of cyclic AMP and progesterone production in rat corpora lutea of different ages. Acta Endocrinol (Copenh). 100:613–622.
7. **Jordan AW, Caffrey JL, Niswender GD**. 1978 Catecholamine-induced stimulation of progesterone and adenosine 3′,5′-monophosphate by dispersed ovine luteal cells. Endocrinology. 103:385–392.
8. **Battista PJ, Poff JP, Deaver DR, Condon WA**. 1987 Biogenic amine regulation of bovine luteal progesterone production *in vivo*. J Reprod Fertil. 80:517–522.
9. **Luck MR, Jungclas B**. 1987 Catecholamines and ascorbic acid as stimulators of bovine oxytocin secretion. J Endocrinol. 114:423–430.
10. **Wheeler AG, Lean J, Walker M**. 1988 Peripheral progesterone concentrations in the luteal-phase ewe: effects of a beta-adrenergic receptor antagonist and two beta 2-adrenergic agonists. J Endocrinol. 116:137–142.
11. **Webley GE, Luck MR, Hearn JP**. 1988 Stimulation of progesterone secretion by cultured human granulosa cells with melatonin and catecholamines. J Reprod Fertil. 84:669–677.
12. **Casper RF, Cotterell MA**. 1984 The effects of adrenergic and cholinergic agents on progesterone production by human corpus luteum *in vitro*. Am J Obstet Gynecol. 148:663–669.
13. **Richard JS, Hedin L**. 1988 Molecular aspects of hormone action in ovarian follicular development, ovulation, and luteinization. Ann Rev Physiol. 50:441–463.
14. **Taylor MJ, Clark CL**. 1988 Stimulatory effect of phorbol diester on relaxin release by porcine luteal cells in culture. Biol Reprod. 39:743–750.
15. **Veldhuis JD, Yoshida K, Dubois IV, Fields MJ**. 1987 Maitotoxin stimulates steroid and peptide hormone secretion by swine luteal tissue. Am J Physiol. 252:E8–E12.
16. **Brunswick B, Muhkopadhay AK, Budnik LT, Bohnet HG, Leidenberger FA**. 1986 Phorbol ester stimulate progesterone production by isolated bovine luteal cells. Endocrinology. 118:743–749.
17. **Wegner JA, Martinez-Zaguilan R, Gillies RJ, Hoyer PB**. 1991 Prostaglandin F2-alpha-induced calcium transient in ovine large luteal cells: II. Modulation of the transient and resting cytosolic free calcium alters progesterone secretion. Endocrinology. 128:929–936.
18. **Hansel W, Alila HW, Dowd JP, Yang X**. 1987 Control of steroidogenesis in small and large bovine luteal cells. Aust J Biol Sci. 40:331–347.
19. **Komorowski LI, Tsang BK**. 1990 Divergent effects of 1-oleoyl-2-acetylglycerol and tumor-promoting phorbol ester on rat granulosa cell steroidogenesis *in vitro*. Biol Reprod. 43:73–79.
20. **Greenwald GS, Terranova PF**. 1986 Follicular selection and its control. In: Knobil E, Neill J, Ewing LL, Markert CL, Greenwald GS, Pfaff DW, eds. The Physiology of Reproduction. New York: Raven Press; 527–445.
21. **Heindel JJ, Berkowitz AS, Philo R, Preslock JP**. 1981 FSH stimulation of cAMP accumulation in hamster Sertoli cells: effects of age and optic enucleation. J Androl. 2:217–221.
22. **Flores JA, Veldhuis JD, Leong DA**. 1990 Follicle-stimulating hormone evokes and increase in intracellular free calcium ion concentrations in single ovarian (granulosa) cells. Endocrinology. 127:3172–3179.
23. **Mayerhofer A, Föhr KJ, Sterzik K, Gratzl M**. 1992 Carbachol increases intracellular free calcium concentrations in human granulosa-lutein cells. J Endocrinol. 135:153–159.
24. **Sterzik K, Grab D, Sasse V, Hütter W, Rosenbusch B, Terinde R**. 1989 Doppler sonographic findings and their correlation with implantation in an *in vitro* fertilization program. Fertil Steril. 52:825–828.
25. **Grynkiewicz G, Poenie M, Tsien RY**. 1985 A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. J Biol

Chem. 260:3440–3450.
26. **Föhr KJ, Scott J, Ahnert-Hilger G, Gratzl M.** 1989 Characterization of the inositol 1,4,5-trisphosphate-induced calcium release from permeabilized endocrine cells and its inhibition by decavanate and p-hydroxymercuribenzoate. Biochem J. 262:83–89.
27. **McAllister JM, Mason JI, Byrd W, Trant JM, Waterman MR, Simpson ER.** 1990 Proliferating human granulosa-lutein cells in long term monolayer culture: expression of aromatase, cholesterol side-chain cleavage and 3 beta hydroxysteroid dehydrogenase. J Clin Endocrinol Metab. 71:26–33.
28. **McGrath JC, Brown CM, Wilson VG.** 1989 Alpha-adrenoceptors: a critical review. Med Res Rev. 9:407–533.
29. **Rajkumar K, Chedrese PJ, Ly H, Murphy BD.** 1991 Protein kinase C, an endogenous regulator of hormone-induced cyclic AMP induction in porcine luteal cells. J Endocrinol. 130:273–280.
30. **Chik C, Ho AK.** 1989 Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. Prog Biophys Mol Biol. 53:197–203.
31. **Stoelk E, Chegini N, Lei ZM, Rao ChV, Bryant-Greenwood G, Sanfilippo J.** 1991 Immunocytochemical localization of relaxin in human corpora lutea: cellular and subcellular distribution and dependence on reproductive state. Biol Reprod. 44:1140–1147.
32. **Sherwood OD.** 1988 Relaxin. In: Knobil E, Neill J, Ewing LL, Markert CL, Greenwald GS, Pfaff DW, eds. The Physiology of Reproduction. New York: Raven Press; 585–673.
33. **Almers W.** 1990 Exocytosis. Annu Rev Physiol. 52:607–624.
34. **Rojas FJ, Asch RH.** 1985 Effects of luteinizing hormone-releasing hormone agonist and calcium upon adenylate cyclase activity of human corpus luteum membranes. Life Sci. 36:841–850.
35. **Burden HW.** 1985 The adrenergic innervation of mammalian ovaries. In: Catecholamines as Hormone Regulators. Ben-Jonathan N, Bahr JM, Weiner RI, eds. New York: Serono Symposium Publications, Raven Press, 261–278.
36. **Lara HE, Belmar J.** 1991 Release of norepinephrine from the cat ovary: changes after ovulation. Biol Reprod. 44:752–759.
37. **Dennefors B, Hamberger L, Hillensjö T, et al.** 1983 Aspects concerning the role of prostaglandins for ovarian function. Acta Obstet Gynecol Scand [Suppl]. 113:31–41.
38. **Grob HS.** 1972 Effects of abdominal ovarian denervation on vaginal opening, estrus and ovarian histology in mice. Fed Proc. 31:265.
39. **Grob HS, Brink CE.** 1973 Effects of exogenous norepinephrine on the ovaries of hypophysectomized mice. Fed Proc. 32:213.
40. **Hartshorne GM.** 1990 Subpopulations of granulosa cells within the human ovarian follicle. J Reprod Fertil. 89:773–782.