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Effect of Oxytocin on Free Intracellular Ca^{2+} Levels and Progesterone Release by Human Granulosa-Lutein Cells*

ARTUR MAYERHOFER, KARL STERZIK, HEIKE LINK, MARTIN WIEMANN, AND MANFRED GRATZL

Abteilung Anatomie und Zellbiologie and Abteilung Vergleichende Neurobiologie (M.W.), Universität Ulm, and Universitätsfrauenklinik Ulm (K.S.), Prittwitzstraße 43, D-7900 Ulm, Germany

ABSTRACT

Oxytocin and its receptor are found in the corpus luteum in a variety of species, including the human. In the present study we used fura-2 microfluorimetry to investigate whether activation of the oxytocin receptor of cultured human granulosa-lutein cells causes intracellular calcium (Ca^{2+}) signals and affects progesterone release. Although after 1 day in culture, cells were not responsive to oxytocin, the number of responsive cells increased steadily during the first 3 days in culture, reaching a maximum on days 4 and 5 (59–66%) and then declined again until day 8. Effective oxytocin concentrations were apparently independent of the culture day, and concentrations as low as 10 nmol/L increased intracellular free Ca^{2+} levels from 70–140 nmol/L (basal levels) to maximal peak levels of 800 nmol/L. The oxytocin-induced Ca^{2+} signal was not affected by removal of extracellular Ca^{2+} with EGTA. Moreover, depletion of intracellular Ca^{2+} stores by ionomycin treatment rendered the cells unresponsive to oxytocin, pointing also at the intracellular source of the oxytocin-inducible Ca^{2+} signal. Interestingly, after one single stimulation with oxytocin, cells became refractory to additional stimuli, and only extremely high concentrations of oxytocin induced a second increase in intracellular free Ca^{2+}. To examine the possible effects of oxytocin on progesterone release by cultured cells, we incubated cells on culture day 2 (20% responsive cells in the fura measurements) and culture day 5 (66% responsive cells in the fura measurements) for 24 h with oxytocin (10 nmol/L) and hCG (10,000 IU/L). Although hCG significantly stimulated progesterone release, oxytocin alone was without a stimulatory effect on either day. However, a significant augmentation of the effect of hCG on progesterone release was found in incubations of cells on day 5. Interestingly, the effects of hCG also included stimulation of oxytocin release by cultured granulosa-lutein cells into the culture medium, as determined by RIA. In summary, our data indicate the presence of a functional oxytocin receptor on human granulosa-lutein cells that is linked to Ca^{2+} as a second messenger released from intracellular Ca^{2+} stores. The number of oxytocin-responsive cells increases during differentiation in culture. Moreover, oxytocin release induced by hCG and a stimulatory effect of oxytocin on the hCG-induced progesterone production during the period of maximal responsiveness of cultured cells were found. We, therefore, propose that oxytocin may have autocrine and/or paracrine functions in human granulosa-lutein cells, including fine-tuning of progesterone release. (*J Clin Endocrinol Metab* **77:** 1209–1214, 1993)

THE SYNTHESIS of oxytocin in the corpus luteum of the ovary is well established and occurs in a variety of species (1–3; for review, see Ref. 4), including the human (5–8). Moreover, indirect evidence for the presence of oxytocin receptors in human granulosa-lutein cells was provided by a study that showed inhibitory effects of oxytocin on FSH-stimulated estradiol secretion (9). Using microdialyzed human corpora lutea, paracrine effects of oxytocin on steroid production were reported. Oxytocin stimulated both progesterone and estradiol release in the young human corpora lutea examined (10). Besides these reports, which give indirect evidence for functional oxytocin receptors in the human ovary, more recently their presence was proven by the detection of oxytocin receptor mRNA in the human ovary (11). However, the signal transduction pathway of the oxytocin receptor in the human corpus luteum has not been examined, and there is no information available concerning whether the expression of oxytocin receptors changes during differentiation. We, therefore, tried to address these points in the present study. We investigated whether activation of oxytocin receptors can cause elevations of intracellular free calcium concentrations in human granulosa-lutein cells in a manner similar to that we reported previously in cultured human myometrial cells (12) and rat pituitary corticotrophs (13, 14). In addition, we examined the role of oxytocin in progesterone release, and the effect of hCG exerted on the release of oxytocin by cultured human granulosa-lutein cells.

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Address all correspondence and requests for reprints to: Prof. Dr. M. Gratzl, Abteilung Anatomie und Zellbiologie der Universität Ulm, D-89069 Ulm, Germany.

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Materials and Methods

Materials

Fura-2 and fura-2/AM (pentaacetoxymethyl ester) were purchased from Calbiochem (La Jolla, CA), and EGTA and dimethylsulfoxide from Fluka (Neu Ulm, Germany). Fetal calf serum was obtained from Gibco (Berlin, Germany), and human oxytocin from Boehringer (Mannheim, Germany). All other chemicals, including Dulbecco's Modified Eagle's Medium, Ham's F-12 medium, and ionomycin were obtained from Sigma (Munich, Germany).

Methods

Culture of human granulosa-lutein cells. The procedure has been described previously in detail (15, 16). Human granulosa cells for this study were isolated from follicular fluid obtained from a total of 52 women in connection with the *in vitro* fertilization program at the Universitätsfrauenklinik Ulm (pooled cells of maximally 4 patients). For the calcium measurements in the present study, we used pools of aspiration fluids (40 patients). For measurements of progesterone secretion, cells from an

additional 9 women were used (3 pools of 3 women each); for the measurements of oxytocin production, we used a pool of cells from 3 women. The cells were washed with Dulbecco's Modified Eagle's Medium-Ham's F-12 (1:1) and seeded in the same medium with 10% fetal calf serum in 60-mm Falcon (Becton-Dickinson, Heidelberg, Germany) culture dishes. Cells were kept in an incubator at a humidified atmosphere with 5% CO_2 at 37 C. After 24 h, media were replaced, and all nonadherent cells (mainly red blood cells) were removed by gentle washing. Subsequently, medium was changed every 2 days. Cells were used for calcium measurements on days 1, 2, 3, 4, 5, 6, and 8 (total of 197 cells) after plating. Granulosa cells in culture as well as *in vivo* undergo luteinization (17). Luteinization of the cells is revealed by their ability to produce progesterone (Sterzik, K., and A. Mayerhofer, unpublished) and their typical morphology (18).

Measurements of oxytocin release. To obtain an estimate of whether the granulosa-lutein cells used in our *in vitro* system produce oxytocin, pooled cells from three women were incubated for 24 h, starting after the first day in culture, with or without hCG (10,000 IU/L). A specific oxytocin antiserum (final dilution, 1:70,000; Ferring, Kiel, Germany) was used for determination of oxytocin by RIA. Antiserum showed cross-reactivies of less than 0.01% with arginine vasotocin, arginine vasopressin, lysine vasopressin, somatostatin, and LH-releasing hormone, 0.001% with ACTH, and 2% with neurophysin. Oxytocin from Bachem (Heidelberg, Germany) was used to generate standards for the RIA. The tracer, [^{125}I]oxytocin, was prepared by the chloramine-T method (19). Aliquots of cell culture supernatants were filtered at 4 C using an Ultrafree-MC 30,000 separation device (Millipore, Eschborn, Germany) to remove molecules larger than 30,000 daltons. Recovery of oxytocin from the filtrates exceeded 95%, as determined with [^{125}I]oxytocin. Samples were subsequently shock-frozen and lyophylized. Immediately before use they were reconstituted in a 3- to 4-fold volume of RIA buffer (0.05 mol/L sodium phosphate, 1% NaCl, 0.1% NaN_3, 0.25% EDTA, 0.1% BSA, and 1% porcine serum, pH 7.2). The detection limit was 1 fmol/tube. All samples were run in triplicate, and intra- and interassay coefficients of variations were 10% and 12%, respectively.

Microscopic fluorescence measurements of single cells. As reported previously (15), cells were loaded with fura-2/AM in culture medium without serum (15–30 min at 37 C). Fura-2 acetoxymethyl ester (fura-2/AM, dissolved in dimethylsulfoxide) was added from a stock to give a final concentration of 1.5 μmol/L fura-2/AM and 0.1% dimethylsulfoxide. The cells were then washed with solution A (140 mmol/L NaCl, 4.7 mol/L KCl, 1.2 mol/L KH_2PO_4, 1.2 mol/L $MgSO_4$, 1 mol/L $CaCl_2$, 0.5 mol/L ascorbic acid, 11 mol/L glucose, and 15 mol/L piperazine-N,N'-bis(2-ethane sulfonic acid), pH 7.2) and used for Ca^{2+} measurements within 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+}. Fluorescence measurements were made with the Zeiss Microscope Photometer System (FFP, Zeiss, Oberkochen, Germany), as described previously (15). Ratios of the recordings at 340 and 380 nm were determined as previously described (20).

Test substances were either added with a motor-driven syringe (Microlab P system, speed setting 9, Hamilton, Reno, NV) connected with tubings or by hand pipetting. 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. All consecutive treatments with several test substances were carried out by handpipetting. The concentrations reported in the following are undiluted concentrations in the syringe (stock) before adding them to the cells.

Measurements of progesterone release: incubation experiments. Two 24-h incubation experiments were performed, each with pooled cells harvested from 3 women, which were seeded onto 12 culture dishes each (3 dishes/treatment). Exp I was started 24 h (during culture day 2) after plating. Exp II was started on the morning of the fifth day after plating. In all cases, cells were washed and exposed to fresh culture medium (control groups), medium containing 10 nmol/L oxytocin or hCG (at 10,000 IU/L), as well as oxytocin and hCG in combination. The total volume per dish was 3 mL. After 6 h, the supernatants were collected and frozen until measurement of progesterone using routine RIA protocols at the Universitätsfrauenklinik Ulm, as previously described (15, 16). Cells were harvested, and protein concentrations were determined with the help of the BCA bicinchoninic acid (Pierce, Rockford, IL) method. The progesterone produced was expressed as nanomoles per L/mg cellular protein.

Statistics. Data from progesterone measurements were evaluated by analysis of variance, followed by Scheffe's F test and/or Fisher's test.

Results

Calcium measurements

The basal values of intracellular free Ca^{2+} ranged between 70–140 nmol/L, as described previously (n = 197 individual cells; see Figs. 2–6 before stimulation). The basal Ca^{2+} value was stable, and spontaneous changes in intracellular free Ca^{2+} were not observed (observation periods ranging from 2–20 min). Addition of oxytocin to cultured human granulosa-lutein cells within 2–15 s increased intracellular free Ca^{2+} levels in a fraction of these cells, depending on the culture day. Although after 1 day in culture, cells were not responsive, the number of responsive cells increased steadily during the first 3 days in culture, reaching a maximum on days 4 and 5 (59–66%), and then declined again until day 8 (Fig. 1). Differences in the concentrations of oxytocin effectively raising Ca^{2+} apparently were not dependent on the culture day, and concentrations as low as 10 nmol/L increased intracellular free Ca^{2+} levels to maximal peak levels of about 800 nmol/L (Fig. 2). The oxytocin-induced Ca^{2+} signal was not affected by removal of extracellular Ca^{2+} with EGTA (Fig. 3). Occasionally, a smaller additional Ca^{2+} transient was observed (Fig. 4). Moreover, depletion of intracellular Ca^{2+} stores by ionomycin treatment (additional 14 cells) in the absence of extracellular Ca^{2+} rendered the cells unresponsive to oxytocin (Fig. 5). These results indicate that oxytocin caused the release of Ca^{2+} from an intracellular source. Interestingly, after a single stimulation with low concentrations of oxytocin (10 nmol/L), cells became refractory to additional stimuli, and only extremely high concentrations of oxytocin (100 μmol/L) induced a second increase in intracellular free Ca^{2+} (Fig. 6).

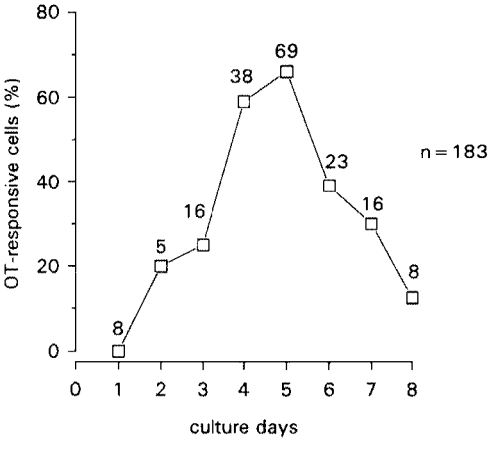

FIG. 1. Percentages of human granulosa-lutein cells responding to oxytocin by increasing intracellular free Ca^{2+} levels: dependence on time in culture. The number of cells tested per time point and total numbers of cells are indicated.

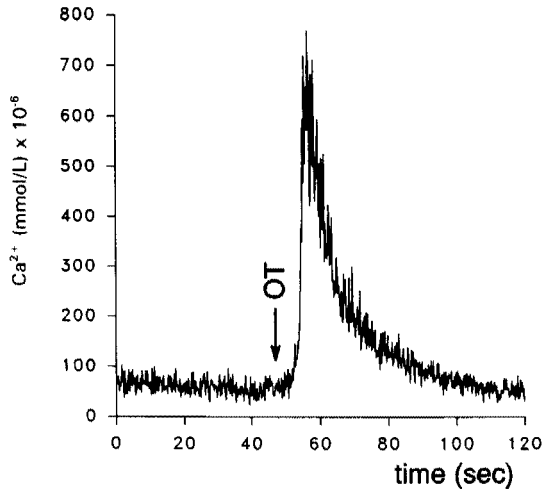

FIG. 2. Intracellular free Ca^{2+} transient in a single human granulosa-lutein cell (culture day 3) after adding oxytocin. Within a few seconds after adding 10 nmol/L oxytocin (OT; concentrations inside the pipette) a rapid increase in free intracellular Ca^{2+} was seen, after which Ca^{2+} levels returned to basal values. This specific experiment was successfully repeated twotimes. Note that Figs. 2–6 are representative results for several experiments, as indicated.

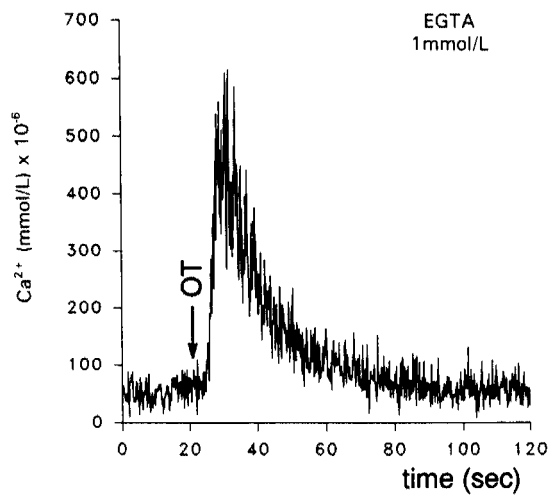

FIG. 3. The effect of oxytocin on a single human granulosa-lutein cell (culture day 3) was not abolished by performing the experiments in the absence of extracellular Ca^{2+}. As in Fig. 2, a rapid increase in free intracellular Ca^{2+} was seen after adding oxytocin (OT; 1 μmol/L; all concentrations inside the pipette). This specific experiment was successfully repeated four times.

Progesterone release

To examine the effects of oxytocin on progesterone release by cultured cells, we incubated cells on days 2 (20% responsive cells; Exp I) and 5 (66% responsive cells; Exp II) for 24 h with oxytocin and hCG (Fig. 7). Basal progesterone release was much higher after 2 days in culture than after 5 days. On either day, hCG significantly stimulated progesterone release, but oxytocin alone was without a stimulatory effect. Only after 5 days in culture did a statistically significant augmenting effect of oxytocin on hCG-stimulated progesterone production became evident (Fig. 7B).

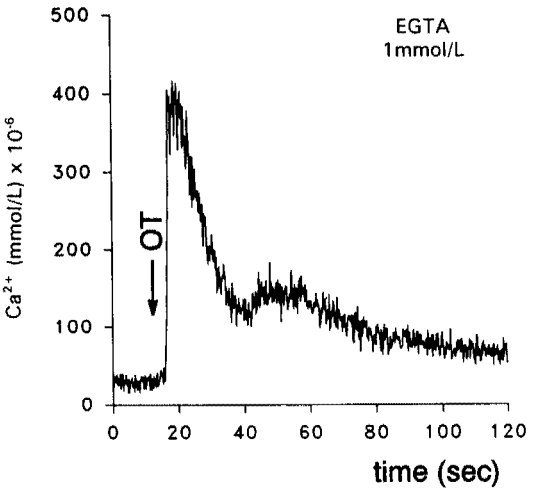

FIG. 4. The effect of a higher concentration of oxytocin (100 μmol/L) on a single human granulosa-lutein cell (culture day 5) in the absence of extracellular Ca^{2+}. Note the second smaller Ca^{2+} transient and the delayed return to basal values. This experiment was successfully repeated 10 times.

Oxytocin release

Human granulosa-luteal cells were found to release measurable amounts of oxytocin into the culture medium only after the addition of hCG, whereas oxytocin was below the detection limit under control conditions (two wells). The values in two wells obtained after hCG stimulation (10,000 IU/L) were 12.6 and 13.8 fmol oxytocin/mg cellular protein released·24 h from days 1–2 of culture.

Discussion

The present study shows that the oxytocin receptors of cultured human granulosa-lutein cells are functionally linked to increases in cytosolic free Ca^{2+}, which is mobilized from intracellular stores. We provide evidence that the numbers of cells endowed with functional receptors for oxytocin increases during differentiation in culture, and that oxytocin, released by human granulosa-lutein cells, can act as a paracrine/autocrine regulator of hCG-induced progesterone secretion.

In our cell culture system, human granulosa-lutein cells secrete oxytocin if stimulated with hCG. Previously, the secretion of oxytocin by human granulosa cells in a serum-free culture system was reported to be in the range of about 15 pmol/L·10^5 cells (21). In contrast to this study, we seeded our cells in clusters (avoiding enzymatic dispersion), rather than as single cells, and used fetal calf serum-supplemented medium, rather than medium without serum. Therefore, the values obtained in our study (expressed per mg cellular protein) can not be readily compared with the data from the literature. We were unable to detect oxytocin in the absence of hCG in the culture supernatants. This could be due to the fact that we used serum, and serum has been described to inhibit oxytocin secretion by cultured bovine granulosa cells (22). Although a number of factors can stimulate oxytocin secretion of granulosa-lutein cells [*e.g.* prostaglandins (2), acetylcholine (23), insulin, insulin-like growth factor-I, and

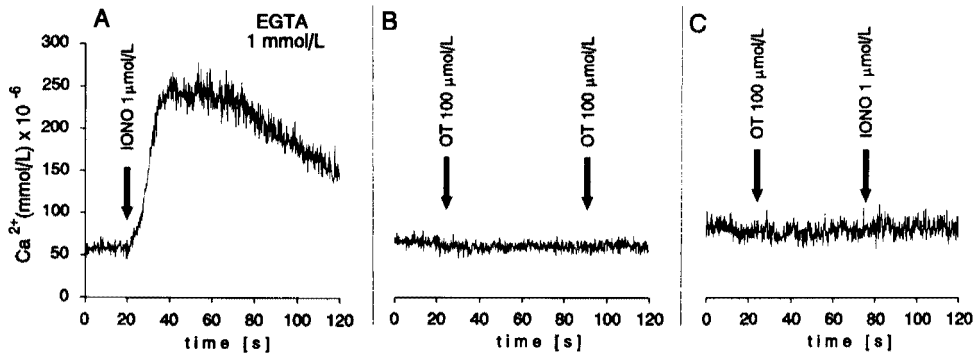

FIG. 5. Effects of ionomycin on oxytocin-triggered Ca^{2+} transients. The time course of changes in intracellular free Ca^{2+} levels in response to 1 µmol/L ionomycin (IONO; 1 µmol/L) of a single human granulosa-lutein cell (5 days in culture) is depicted, which is representative of 14 experiments. Note that calcium in the medium was replaced by 1 mmol/L EGTA. This cell shows a strong response to the addition of ionomycin (A). After approximately 3 min (B), the Ca^{2+} levels returned to baseline. Increasing concentrations of oxytocin (OT) were subsequently given to the cells (B and C), but were not effective to induce an increase in Ca^{2+} levels.

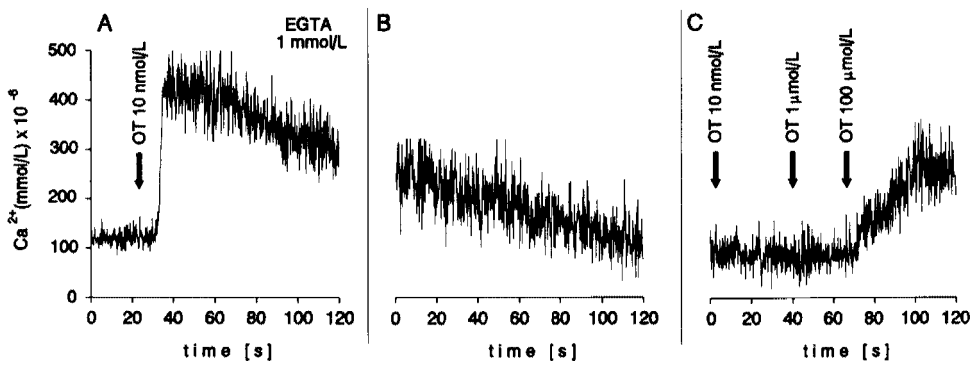

FIG. 6. Human granulosa-lutein cells become insensitive to oxytocin. A, The effects of 10 nmol/L OT. Note that an increased level of approximately 500 nmol/L Ca^{2+} was achieved, after which the Ca^{2+} levels slowly returned to basal levels (B). After the first stimulation, only 10,000 times higher concentrations of oxytocin (C) were able to cause a small elevation of intracellular Ca^{2+} levels. Intervals between A and B are approximately 1 min; those between B and C are approximately 5 min. Cells were cultured for 5 days. The striking desensitization effect of OT was seen in basically all cells tested in the present study.

gonadotropins (see references in Ref. 24)], to our knowledge, hCG is not among them. This situation in human granulosa-lutein cells is, thus, comparable with the stimulation of oxytocin by cultured rat Leydig cells in the presence of LH (25). 17β-Estradiol stimulation of oxytocin synthesis by bovine granulosa cells has recently been reported to depend on the stage of the cycle (26), and estradiol can up-regulate oxytocin gene expression (27, 28). However, in our *in vitro* system, this is unlikely to be responsible for such an effect, as we were unable to detect significant amounts of estradiol in the medium of our cell cultures (levels were usually <14 pmol/L) (Mayerhofer, A., and K. Sterzik, unpublished). Thus, regulation of the biosynthesis and release of oxytocin by cultured human granulosa-lutein requires further investigation and confirmation.

The main novel finding of this study is that granulosa-lutein cells are not only the producers, but also the targets, for oxytocin and that they responded to oxytocin by acute increases in intracellular Ca^{2+}. However, the oxytocin-induced Ca^{2+} transient was not evokable in all cells and was strongly dependent on the culture day. Freshly cultured cells did not respond at all, whereas maximal responsiveness was observed around day 5, after which it declined again. It is possible that this increase in functional oxytocin receptors is closely correlated with the functional and differential state of granulosa-lutein cells. Also, the endogenous production of oxytocin by the cells could contribute to this phenomenon by regulating the oxytocin receptor. Oxytocin secretion by bovine granulosa cells reaches a maximum on the third or fourth day in culture and declines thereafter (see Ref. 22 for references). Unfortunately, we do not have comparable data due to the fact that basal levels of oxytocin were beyond the detection limits of our assay.

The culture time had no apparent influence on the nature of the oxytocin-inducible Ca^{2+} signal itself. Even in the absence of extracellular Ca^{2+}, oxytocin caused a Ca^{2+} transient in responsive cells, indicating the involvement of intracellular Ca^{2+} stores. Additional evidence for the nature of the intracellular source of Ca^{2+} is provided by experiments with ionomycin (15, 29, 30). Thus, depletion of intracellular Ca^{2+} stores (by the ionophore ionomycin) completely abolished the response of the cells upon oxytocin stimulation. To our knowledge, oxytocin exerts its effect via stimulation of phospholipase-C in the human myometrium (12) and rat pituitary (13, 14). A number of other receptors (*e.g.* muscarinic receptors and α-adrenergic receptors on human granulosa-lutein cells (15, 31) are also coupled to phospholipase-C, which, after activation, hydrolyzes phosphatidylinositol-4,5-bisphosphate, which then generates diacylglycerol and inositol-1,4,5-trisphosphate (32). The latter causes an increase in cytosolic free Ca^{2+} by releasing intracellularly stored Ca^{2+}. Our results allow the conclusion that this is also the case for oxytocin in human granulosa-lutein cells.

Oxytocin alone under the conditions of our cell culture

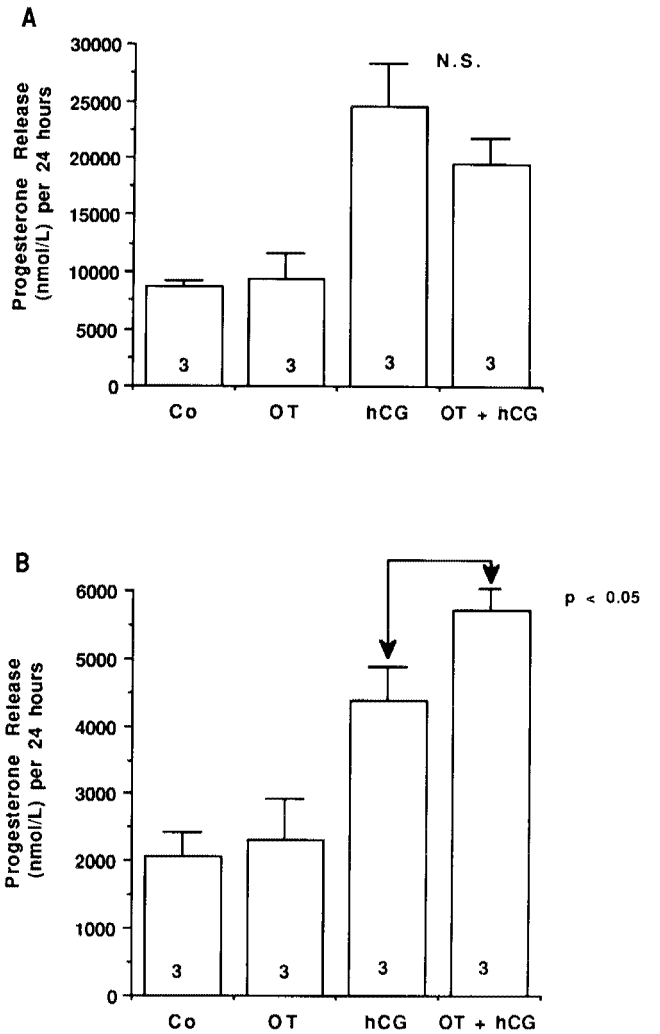

FIG. 7. Effects of oxytocin and hCG on progesterone release. A, Results of 24-h incubations of granulosa-lutein cells with oxytocin and hCG (performed on culture day 2; Exp I). B, Results of 24-h incubations of granulosa-lutein cells with oxytocin and hCG (performed on culture day 5; Exp II). The release of progesterone into the medium is expressed as nanomoles per L/mg protein. For each experiment, the pooled cells of three women were used, and each treatment group represents triplicate experiments (three wells per treatment). Values are the mean ± SEM. Note the important statistically significant differences in B. Co, Basal release; OT, oxytocin (10 nmol/L); hCG, 10,000 IU/L hCG. See *Materials and Methods* for details. The experiment was repeated with a shorter (6-h) incubation time and gave comparable results.

system did not affect progesterone release; however, if combined with hCG, the physiological stimulus of progesterone secretion (33), a small, but statistically significant, augmentation of hCG-induced progesterone release was measured at a time point when most cells showed an oxytocin-inducible increase in intracellular Ca^{2+}. These results are in certain respects comparable with those of a study that reports lack of stimulation of steroid hormone secretion by oxytocin alone (9). However, in a certain number of cases, oxytocin decreased the FSH-stimulated secretion of estradiol. The culture conditions reported were different from the ones in the present study, and therefore, the results are not directly comparable [we did not add androgens to the culture medium and did not detect significant levels of estradiol in the medium (Mayerhofer, A., and K. Sterzik, unpublished data)]. In contrast, using microdialysed young human corpora lutea (early luteal phase days 4–8) instead of cell cultures, oxytocin in the micromolar range has recently been reported to stimulate progesterone and estradiol release (10). These investigators have concluded that the stimulatory effect of oxytocin on progesterone secretion is indirect and mediated by estradiol, as judged by additional experiments using tamoxifen (10). The discrepancies between the micodialysis study and the studies performed in granulosa-lutein cell culture are obvious. We and others (9) did not observe stimulatory effects of oxytocin alone in cultures. Moreover, low levels of estradiol in our cultures appear to rule out the possibility that oxytocin stimulates progesterone via estradiol. Our data indicate a stimulatory concerted effect of hCG and oxytocin on progesterone release. Interestingly, hCG-induced production of progesterone was inhibited in the presence of agonists of the α-adrenoreceptor in the same culture system (31). It remains to be elucidated whether such concerted actions occur within the cells at a postreceptor level and/or are the result of a paracrine cooperation between neighboring cells.

In conclusion, the present and previous studies (15, 31) have shown that single cell Ca^{2+} measurements in a defined culture system of human granulosa-lutein cells in combination with release experiments are useful tools to examine functional receptor-mediated effects of paracrine/autocrine factors on these cells.

Acknowledgments

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