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Carbachol increases intracellular free calcium concentrations in human granulosa-lutein cells

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ABSTRACT

We investigated whether the stimulation of human granulosa-lutein cells with muscarinic and nicotinic receptor agonists can cause increases in intracellular free calcium (Ca^{2+}), using Fura-2 microfluorimetry. The addition of carbachol (a non-selective muscarinic and nicotinic receptor agonist) to cultured human granulosa-lutein cells increased intracellular free Ca^{2+} levels. Concentrations as low as 10 nmol/l were effective. In contrast, nicotine did not evoke elevations of intracellular free Ca^{2+}. Basal Ca^{2+} levels ranged around 70–140 nmol/l and maximal, carbachol-induced peaks reached 1·1 μmol/l. The carbachol-induced Ca^{2+} signal was abolished after preincubation of the cells with the muscarinic receptor antagonists quinuclidinyl benzilate or atropine, but it was not affected by removal of extracellular Ca^{2+}. Further evidence for the involvement of intracellular Ca^{2+} stores is provided by experiments in the absence of extracellular Ca^{2+}. While thapsigargin (a blocker of ATP-driven Ca^{2+} uptake by intracellular stores) and ionomycin (an ionophore by which Ca^{2+} is released from intracellular stores) evoked small Ca^{2+} transients, cells pretreated with these agents did not respond to carbachol any more. These data suggest the presence of a functional muscarinic receptor on human granulosa-lutein cells and imply the involvement of intracellular Ca^{2+} stores during the cellular response. These results also suggest the participation of the nervous system, acting through muscarinic receptors, in the control of the function of human granulosa-lutein cells.

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INTRODUCTION

The support of luteinizing hormone (LH) or human chorionic gonadotrophin (hCG) is essential for the survival and function of the primate corpus luteum (Hsueh, Adashi, Jones & Welsh, 1984; Niswender & Nett, 1988). However, there is also increasing evidence for the functional involvement of ovarian innervation by adrenergic and cholinergic nerves (see Burden, 1978; Burden & Lawrence, 1978, Tsafriri, 1988; Luck, 1990). Stimulation by acetylcholine of bovine granulosa cells, which were undergoing luteinization in culture, has been reported to cause secretion of oxytocin and progesterone (Luck, 1990) and the muscarinic receptor antagonists scopolamine and atropine have been shown to inhibit the effect of acetylcholine (Luck, 1990). The author therefore proposed the presence of muscarinic receptor on bovine granulosa-lutein cells. Based on these results, we have examined, in the present study, whether cultured human granulosa-lutein cells possess a muscarinic receptor coupled to intracellular Ca^{2+}.

MATERIALS AND METHODS

Materials

Fura-2 and Fura-2/penta-acetoxymethyl ester (AM) were purchased from Calbiochem (La Jolla, CA, U.S.A.), EGTA and DMSO from Fluka (Neu Ulm, Germany) and medium DMEM-F12 from Sigma (Munich, Germany). Fetal calf serum (FCS) was bought from Gibco (Berlin, Germany). Quinuclininyl benzilate (QNB) was a generous gift from Thomae Pharmaceutical Company (Biberach, Germany). All other chemicals including atropine, thapsigargin and ionomycin nicotine-di-tartrate-HCl were from Sigma.

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Methods

Culture of human granulosa-lutein cells

Human granulosa cells were isolated from the follicular fluid (after the cumulus oopherus cells and the oocyte were separated) obtained from 23 women undergoing aspiration of follicular fluid for in-vitro fertilization at the Universitätsfrauenklink Ulm. The project was approved by the local ethical committee and the women gave written consent. Follicular growth was stimulated prior to follicular puncture by treatment of the women with human menopausal gonadotrophin. Follicular growth was monitored by ultrasound and daily measurements of oestradiol, progesterone and LH. Ovulation was induced with 10 000 IU hCG as the follicles reached a diameter of 17 mm. Follicular aspiration was performed 32–36 h after the induction of ovulation under sonographic control in all women who showed a continuous rise in serum levels of oestradiol (Sterzik, Grab, Sasse *et al.* 1989). Cells were mechanically dispersed by repeated aspiration into a 5 ml pipette and through a 20 gauge needle. Follicular fluid was subsequently mixed with dextran T250 (4·5%, w/v) in 0·9% (w/v) NaCl; 5:1; Roth, Karlsruhe, Germany) and placed in an incubator (37 °C) for 30 min to allow the contaminating red blood cells to settle. Granulosa cells in the supernatant were removed and centrifuged (800 r.p.m., approximately 1000 ***g***; Heraeus Digifuge; 3 min). The resulting pellets were repeatedly washed with DMEM:Ham's F12 (1:1; v/v) and centrifuged. Cells were plated with DMEM:Ham's F12 (1:1) with 10% fetal calf serum in 60 mm Falcon (Becton-Dickinson, Heidelberg, Germany) culture dishes and kept in an incubator at a humidified atmosphere with 5% CO_2 at 37 °C. One day after plating, media were replaced and all non-adherent cells (mainly red blood cells) removed by careful washing. Subsequently, medium was changed every 2 days and cells were used 2 and 4 days after plating. Granulosa cells in culture, as well as *in vivo*, undergo luteinization (see Webley, Luck & Hearn, 1988). Luteinization of the cells in the present study is indicated by the ability of the cells to produce progesterone (K. Sterzik & A. Mayerhofer, unpublished results) and the typical morphology of these cells, which resembled the morphology reported previously (McAllister, Mason, Byrd *et al.* 1990).

Fura-2 loading

The cells were loaded with Fura-2/AM (1·5 μmol/l; dissolved in DMSO) in culture medium without serum for 15–30 min at 37 °C. The cells were then washed with solution A (concentrations given 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 Pipes; pH 7·2) and used for Ca^{2+} measurements within 1–3 h. Solution A was modified by adding 1 mmol EGTA/l instead of 1 mmol $CaCl_2$/l to carry out measurements in the absence of extracellular Ca^{2+}.

Microscopic measurements of single cells

Fluorescence measurements were performed using a Zeiss Microscope Photometer System (fast fluorescence photometry; Zeiss, Oberkochen, Germany), which is based on an inverted microscope (Axiovert 35), equipped for epifluorescence. Measurements at 340/10 nm and 380/10 nm were performed. Fluorescence light from an area adjusted to the cell size was collected (usually approximately 400 μm^2). The autofluorescence of unloaded cells (less than 1% of the fluorescence of dye-loaded cells) was ignored. Background was determined in a cell-free area which contributed to about 5–10% of the signal obtained from Fura-2-loaded cells. Cell signals were corrected for 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). Ratio calculations of the 340 nm recording divided by the 380 nm recordings were carried out according to Grynkiewicz, Poenie & Tsien (1985). The calculations are based on the formula:

$$[Ca^{2+}] = K_d \times (R - R_{min}/R_{max} - R) \times \beta$$

(R, ratio of fluorescence [F]; R_{max}, 340 nm/F380 nm (fluorescence ratio at $[Ca^{2+}]$ saturation); R_{min}, F340 nm/F380 nm (fluorescence ratio in the absence of $[Ca^{2+}]$); β = F380 (at zero $[Ca^{2+}]$)/F380 (at $[Ca^{2+}]$ saturation); K_d = 224.nmol/l).

Calibration of the Fura-2 signal

The Fura-2 signals were calibrated with solution B (150 mmol KCl/l, 20 mmol Mops/l, pH 7·2; see Föhr, Scott, Ahnert-Hilger & Gratzl, 1989). Solution B, supplemented with 5 μmol/l of the free acid of Fura 2 and either 5 mmol Ca^{2+}/l or 5 mmol EGTA/l was used to determine R_{max}, R_{min} and β. With the experimental setup, R_{max} was 8·5, R_{min} 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 used (Grynkiewicz *et al.* 1990).

Addition of test substances

The substances were added to the culture dishes with a motor driven syringe (Hamilton microlab P system, speed setting 9) connected with tubings, ending via an Eppendorf comfort tip approximately 3 mm from the cell, or by hand pipetting. All test substances were diluted in solution A (exception: QNB and atropine were dissolved in DMSO; final dilution 0·01%) and were added in a total volume of 50 μl. The concentrations of the test substances given below always refer

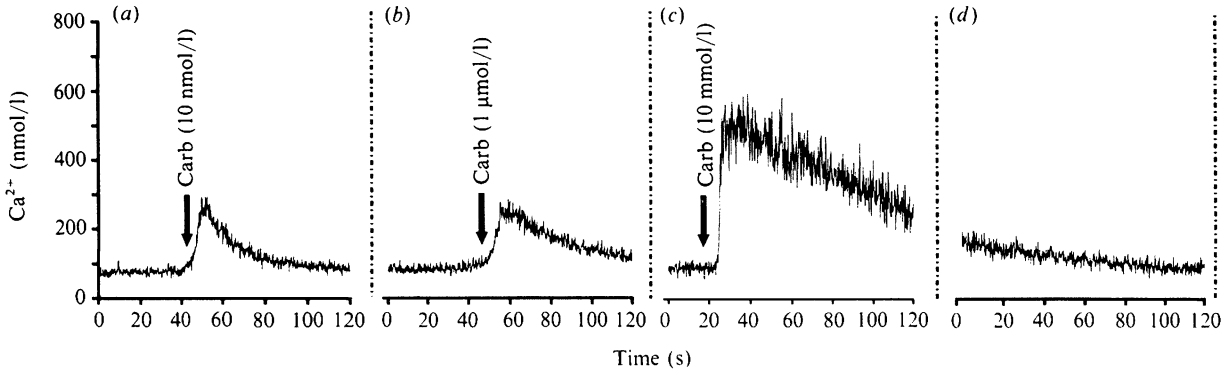

FIGURE 1. Intracellular free Ca^{2+} transient in a single human granulosa-lutein cell after adding increasing doses of carbachol. (*a*) The effects of 10 nmol carbachol/l (Carb; all concentrations inside the pipette). Note that an increased level of approximately 300 nmol Ca^{2+}/l was achieved, after which the Ca^{2+} levels returned to basal. (*b* and *c*) Higher concentrations of carbachol caused higher and sustained elevation of intracellular Ca^{2+} levels, which took several minutes to reach (*d*) baseline level again. Dotted lines between the panels indicate intervals of approximately 5 min (between *a* and *b*, and *b* and *c*) and approximately 30 s (between *c* and *d*). Cells were in culture for 2 days.

to the concentrations of the stock which was added, because the actual effective concentration reaching the cells cannot be given (estimate dilution 1·4).

RESULTS

After Fura-2 loading, the granulosa-lutein cells showed a homogeneous distribution of the dye. The basal values of intracellular free Ca^{2+} ranged between 70 and 140 nmol/l ($n = 183$ individual cells; see Figs 1–4 before stimulation). With the exception of one single cell, the basal Ca^{2+} value was stable and spontaneous changes of intracellular free Ca^{2+} were not observed (observation periods of individual cells ranging from 2 min to 20 min).

The addition of carbachol produced with 1–5 s an increase in the levels of cytoplasmic free Ca^{2+} (with the exception of one cell in all cells tested). The form and maximal peak levels of the Ca^{2+} transients appeared to depend on the carbachol concentration: low concentrations produced a small, single transient characterized by a rapid return to baseline values (Fig. 1*a*). Higher concentrations (mmol range) evoked a greater signal with sustained elevations of Ca^{2+} levels and a prolonged return period to baseline levels (Fig. 1*b*, *c*, and especially *d*). At high concentrations a distinct second, but smaller Ca^{2+} transient was

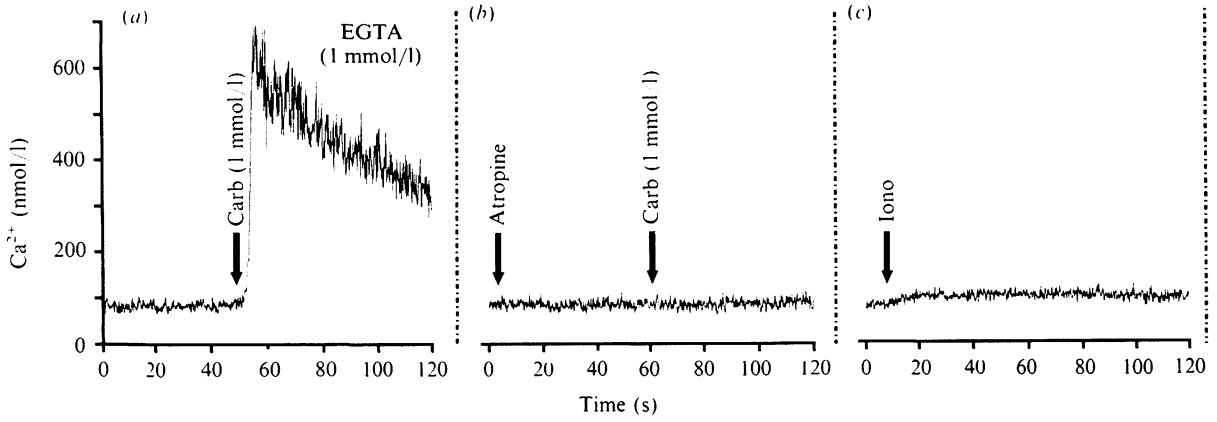

FIGURE 2. The effect of carbachol (Carb) was not abolished by performing the experiments in the absence of extracellular Ca^{2+}. (*a*) The medium contained 1 mmol EGTA/l (see Materials and Methods). Note sustained elevation of the Ca^{2+} levels. (*b*) After adding atropine (100 μmol/l) to the cell, carbachol was not effective any more (time interval between *a* and *b* approximately 3 min). However, (*c*) ionomycin (Iono; 1 μmol/l) was still able to cause a small increase of free intracellular Ca^{2+}. Cells were in culture for 2 days.

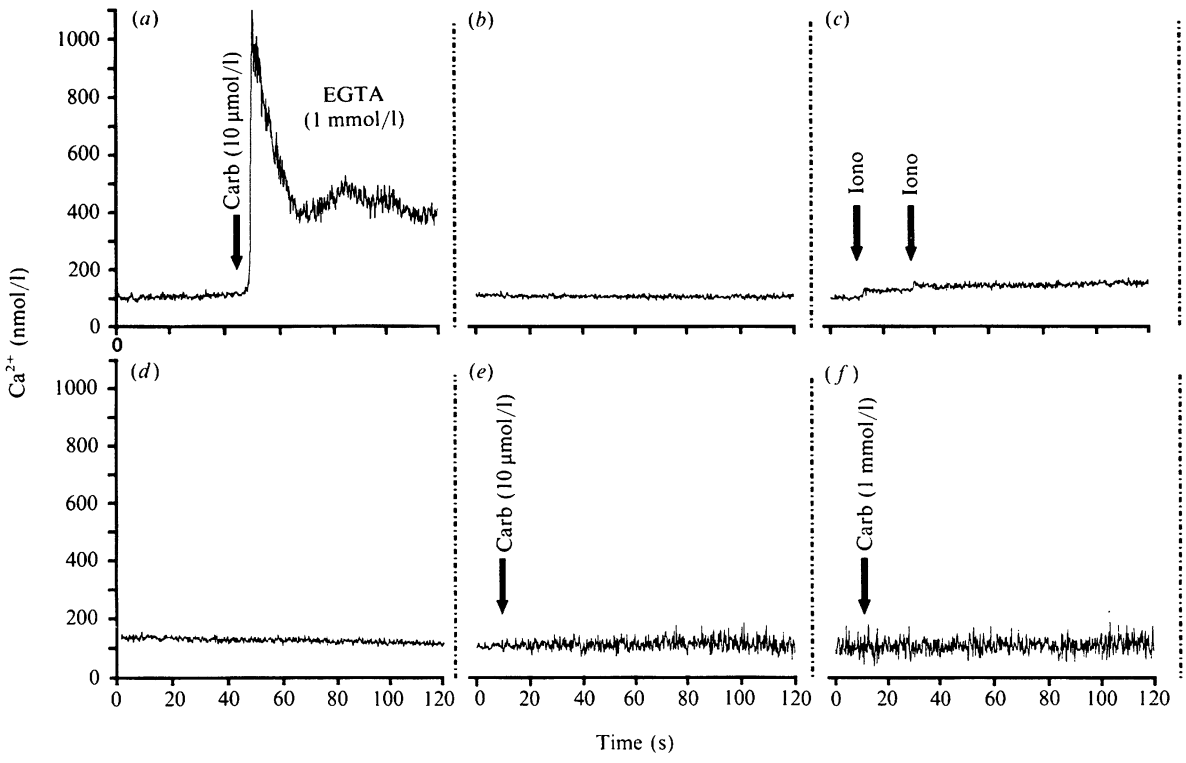

FIGURE 3. Effects of ionomycin on carbachol-triggered Ca^{2+}-transients. (*a*) Time-course of changes in intracellular free Ca^{2+} levels in response to 10 µmol carbachol/l (Carb) of a single human granulosa-lutein cell (2 days in culture) is depicted. Note that calcium in the medium was replaced by 1 mmol EGTA/l. This cell showed an immediate, strong response to the addition of carbachol (initial large peak), followed by a second, smaller Ca^{2+} transient. (*b*) After approximately 3 min the Ca^{2+} levels had returned to baseline. (*c*) Ionomycin (Iono, 1 µmol/l) was given to the cells and caused a small increase in Ca^{2+} levels, which returned to (*d*) basal levels. (*e*,*f*) Carbachol, even at high concentration was subsequently not effective in inducing an increase in Ca^{2+} levels.

occasionally observed (Figs 3 and 4). Intracellular free Ca^{2+} stayed elevated (around 350–400 nmol/l) for 3–5 min and then returned to values near the baseline. Maximal peaks of intracellular free Ca^{2+} reached maximally 1·1 µmol Ca^{2+}/l (Figs 3 and 4). One single cell showed oscillations for 5 min after carbachol (1 mmol/l) was added. Replacing 1 mmol Ca^{2+}/l in the medium by EGTA (1 mmol/l; Figs 2, 3 and 4) did not abolish the carbachol-induced effect on intracellular free Ca^{2+} and did not change the described prolonged return period to baseline values. It should be mentioned that occasionally, as in the presence of Ca^{2+} in the medium, a second peak following the initial transient was also observed (carbachol at 200 µmol/l, 22/23 cells; carbachol at 100 µmol/l, 6/8 cells; carbachol at 10 µmol/l, 10/10 cells; carbachol at 1 mmol/l, 16/16 cells). Preincubation of the cells with the muscarinic antagonist QNB for 30 min prior to carbachol stimulation (carbachol at 10 µmol/l and 100 µmol/l), completely abolished increases in intracellular free Ca^{2+} (QNB at 10 µmol/l, 15/15 cells; QNB at 100 µmol/l, 8/8 cells). In addition, atropine (100 µmol/l) given to the cells prior to carbachol stimulation also prevented the carbachol-induced rise in free intracellular Ca^{2+} (Fig. 2; 9/9 cells examined). Addition of DMSO (0·01%, in which QNB and atropine were diluted) to the cells did not interfere with their ability to respond to carbachol (four cells tested). When, instead of carbachol, nicotine (1 mmol/l) was used, 13/14 tested cells did not respond with elevation of intracellular free Ca^{2+}. To examine the involvement of intracellular Ca^{2+} stores further, additional studies with thapsigargin, which inhibits Ca^{2+} uptake by the intracellular stores (tested at 10 and 100 nmol/l), and the ionophore ionomycin (tested at 1 and 10 µmol/l) were performed in the absence of Ca^{2+} in the medium (Figs 3 and 4). For this purpose carbachol (at 100 µmol/l or 1 µmol/l; Fig. 3*a*) was added to a total of 13 cells. Then ionomycin was added (Fig. 3*c*) and in all 13 cases (at either concentration) caused a small increase of intracellular free Ca^{2+} (Fig. 3*d*) on addition. Ionomycin-treated

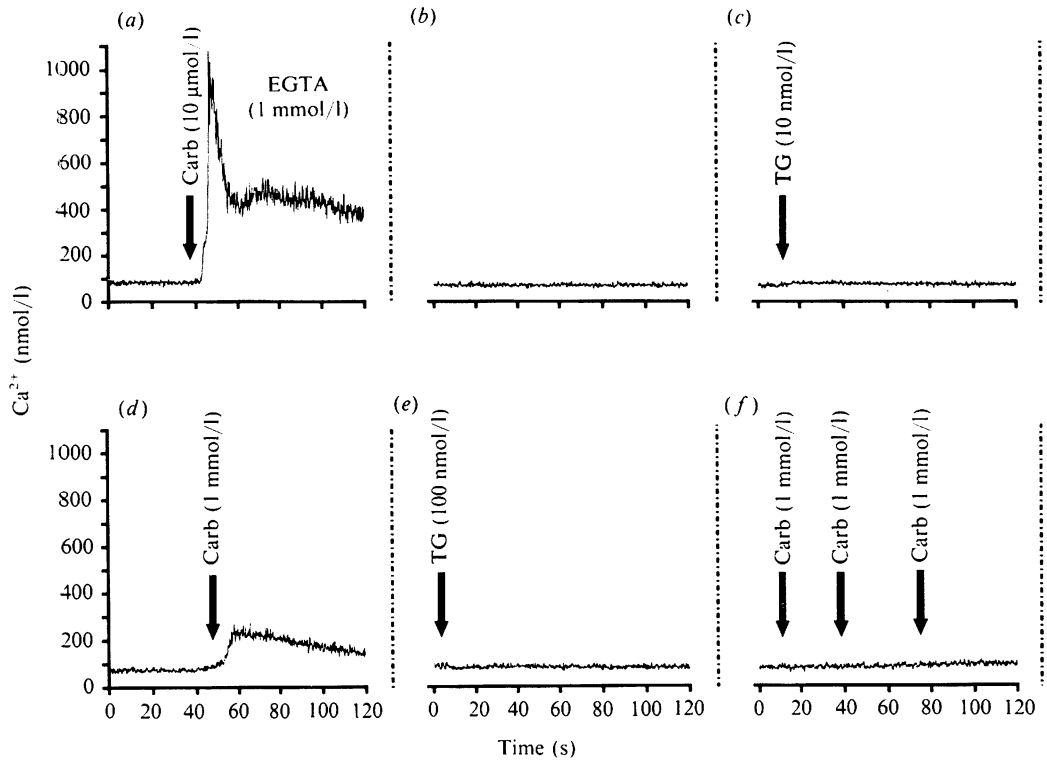

FIGURE 4. Effects of thapsigargin on carbachol-triggered Ca^{2+} transients. The measurement of intracellular free Ca^{2+} in a single human granulosa-lutein cell is seen (compare Fig. 3, cells were cultured for 2 days). (*a*, *b*) The response of this cell to carbachol (Carb; 10 μmol/l) and the experimental protocol was comparable with the experiment depicted in Fig. 3. (*c*) Instead of ionomycin, thapsigargin (TG; at 10 nmol/l) was added. After this treatment, the ability of carbachol to induce a Ca^{2+} increase was partially blocked (note the difference in the height of the signal between *a* and *d*). After (*e*) additional thapsigargin addition (100 nmol/l) the cell (*f*) did not respond to carbachol any more.

cells were subsequently not responsive to carbachol, even at 100 × higher concentration (Fig. 3*e*,*f*). The same protocol was used for thapsigargin (Fig 4; 16 cells tested). However, only the higher dose completely abolished the ability of the cells to respond to carbachol, while the lower dose caused only a gradual decrease of the Ca^{2+} signal (Fig. 4*c*).

DISCUSSION

The present study provides clear evidence, for the first time, for the presence of muscarinic receptors on human granulosa-lutein cells in culture. These receptors are most probably coupled to the phosphoinositide/Ca^{2+} pathway and use intracellular Ca^{2+} stores.

Stimulation of oxytocin and progesterone secretion by bovine granulosa-lutein cells in culture in response to acetylcholine, but not in response to carbachol or nicotine, has been reported by Luck (1990), who, using the muscarinic receptor antagonists atropine and scopolamine, concluded that a muscarinic receptor is probably present on the cells in this species. In contrast to the present work, however, Luck (1990) did not examine the second messenger(s) coupled to the assumed muscarinic receptors. Interestingly, a recent preliminary report in abstract form (Morley, Tsang, Whitfield, & Schwartz, 1991) indicates that, in chicken granulosa cells, carbachol can increase intracellular free Ca^{2+} levels four- to eightfold over basal levels. A detailed study by the same authors has now been published (Morley, Tsang, Whitfield, & Schwartz, 1992): These investigators found that carbachol at a maximally stimulating concentration of 2 mmol/l evoked Ca^{2+} elevations in 85% of all examined cells. In their study, the Ca^{2+} signal appeared to consist of two components: a large initial Ca^{2+} spike, followed by a sustained but lower elevation, with superimposed oscillations and prolonged return to baseline values within several minutes. The initial spike appeared to be derived from release of Ca^{2+} from intracellular stores, because it was not abolished

in the absence of extracellular Ca^{2+} nor by Ca^{2+} channel blockers. In contrast, the sustained elevation is reportedly due to influx of extracellular Ca^{2+} (Morley *et al.* 1992).

The similarities and the discrepancies between the study by Morley *et al.* (1992) and our present study are obvious. Both studies come to the conclusion that granulosa-lutein cells from different species possess functional, Ca^{2+}-activating muscarinic receptors. The main difference is that Morley *et al.* (1992) found that the second component of the carbachol-induced Ca^{2+} signal was due to influx of extracellular Ca^{2+}. We do not come to such a conclusion. We found that even in the absence of extracellular Ca^{2+}, a second Ca^{2+} peak is observed, clearly indicating that solely intracellular Ca^{2+} stores are involved in the response of human granulosa-luteal cells to carbachol. In the present study, the Ca^{2+} signal showed sustained elevations and a second peak only at high carbachol concentrations (in the range as also used by Morley *et al.* 1992), while at lower concentrations the signal obtained lacked these features. Although we have no clear explanation for this, one may speculate that 'over-stimulation' of the cell by high carbachol doses may interfere with the capability of the intracellular stores to take up the excess of intracellular Ca^{2+}. We substantiated the nature of the intracellular source of Ca^{2+} by experiments with thapsigargin and ionomycin (see Fasolato, Zottini, Clementi *et al.* 1991, Zacchetti, Clementi, Fasolato *et al.* 1991). Indeed, blockage of Ca^{2+} uptake by the stores (with thapsigargin) or depletion of the stores (by the ionophore ionomycin) completely abolished the response of the cells upon carbachol stimulation.

Despite the differences of the two studies, our results with human granulosa-lutein cells are in agreement with the reports by Morley *et al.* (1992) in the chicken and Luck (1990) in the bovine species in respect of the presence of a Ca^{2+}-activating muscarinic receptor on granulosa-lutein cells. Currently two muscarinic receptor subtypes, termed M1 and M3 (see Watson & Abbott, 1991) are known to be coupled to phospholipase C, which, after activation, hydrolyses phosphatidylinositol 4,5-biphosphate which generates diacylglycerol and inositol 1,4,5-triphosphate (see Nishizuka, 1986). The latter causes an increase in cytosolic free Ca^{2+} by releasing intracellularly stored Ca^{2+}. Our results permit the conclusion that this is very probably the case in human granulosa-lutein cells, since the effect of carbachol was not affected in the absence of extracellular Ca^{2+} and only disrupting of intracellular stores by thapsigargin and ionomycin (see also Fasolato *et al.* 1991; Zacchetti *et al.* 1991) abolished the carbachol signal.

The ovary is densely innervated by the autonomous nervous system and acetylcholine and its synthesizing enzymes are present in ovarian nerves (Burden, 1978; Burden & Lawrence, 1978). Although Morley *et al.* (1992) did not observe stimulation of progesterone secretion by carbachol in cultures of chicken granulosa cells, Luck (1990) reported that acetylcholine stimulated oxytocin and progesterone secretion by bovine granulosa-luteal cells. Therefore, the functional relevance of our results can be assumed, but this point requires further investigation. The species-differences in the ability of acetylcholine/carbachol to stimulate Ca^{2+}-dependent secretion of products in luteal cells (e.g. by exocytosis; see Almers, 1990) may indicate that this is not the sole function of muscarinic receptor activation and that the nervous system may participate in the regulation of ovarian function in other ways.

In conclusion, we have shown that single cell Ca^{2+} measurement can be used as a tool to examine receptor-mediated effects. Using this method we have obtained evidence for the presence of a functional, Ca^{2+}-mobilizing muscarinic receptor on human granulosa-lutein cells. Clearly, more work is required to elucidate the nature of the muscarinic receptor subtype on human granulosa-lutein cells and details of the involvement of this receptor in the regulation of specific functions within this cell type.

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REFERENCES

Almers, W. (1990). Exocytosis. *Annual Review of Physiology* **52,** 607–673.

Burden, H. W. (1978). Ovarian innervation. In *The Vertebrate Ovary*, pp. 615–638. Ed. R. E. Jones. New York: Plenum Press.

Burden, H. & Lawrence, I. E. Jr. (1978). Experimental studies on the acetylcholinesterase-positive nerves in the ovary of the rat. *Anatomical Record* **190,** 233–242.

Fasolato, C., Zottini, M., Clementi, E., Zacchetti, D., Meldolesi, J. & Pozzan, T. (1991). Intracellular Ca^{2+} pools in PC 12 cells. Three intracellular pools are distinguished by their turnover and mechanism of Ca^{2+} accumulation, storage and release. *Journal of Biological Chemistry* **266,** 20159–20167.

Föhr, K. J., Scott, J., Ahnert-Hilger, G. & Gratzl, M. (1989). Characterization of the inositol 1,4,5-triphosphate-induced calcium release from permeabilized endocrine cells and its inhibition by decavanate and p-hydroxymercuribenzoate. *Biochemical Journal* **262,** 83–89.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *Journal of Biological Chemistry* **260,** 3440–3450.

Hsueh, A. J. W., Adashi, E. Y., Jones, P. B. C. & Welsh, T. H. (1984). Hormonal regulation of the differentiation of cultured ovarian granulosa cells. *Endocrine Reviews* **5,** 76–127.

Luck, M. (1990). Cholinergic stimulation through muscarinic receptors, of oxytocin and progesterone secretion from bovine granulosa cells undergoing spontaneous luteinization in serum-free culture. *Endocrinology* **126,** 1256–1263.

McAllister, J. M., Mason, J. I., Byrd, W., Trant, J. M., Waterman, M. R. & Simpson, E. R. (1990). Proliferating human granulosa-lutein cells in long term monolayer culture: Expression of aromatose, cholesterol side-chain cleavage and 3β-hydroxysteroid dehydrogenase. *Journal of Clinical Endocrinology and Metabolism* **71,** 26–33.

Morley, P., Tsang, B. K., Whitfield, J. F. & Schwartz, J. L. (1991). Carbachol increases chicken granulosa cell intracellular calcium (Ca^{++}), but does not affect cyclic AMP or progesterone production. *Biology of Reproduction* **44,** (Suppl. 1), 119 (Abstract No. 265).

Morley, P., Tsang, B. K., Whitfield, J. F. & Schwartz, J. L. (1992). The effect of muscarinic cholinergic agonists on intracellular calcium and progesterone production by chicken granulosa cells. *Endocrinology* **130,** 663–670.

Nishizuka, Y. (1986). Studies and perspectives of protein kinase C. *Science* **233,** 305–331.

Niswender, G. D. & Nett, T. M. (1988). The corpus luteum and its control. In *The Physiology of Reproduction*, pp. 489–525. Eds E. Knobil, J. Neill *et al.* New York: Raven Press.

Sterzik, K., Grab, D., Sasse, V., Hüttner, W., Rosenbusch, B. & Terinde, R. (1989). Doppler sonographic findings and their correlation with implantation in an *in vitro* fertilization program. *Sterility and Fertility* **52,** 825–828.

Tsafriri, A. (1988). Local nonsteroidal regulation of ovarian function. In *The Physiology of Reproduction*, pp. 527–565. Eds E. Knobil, J. Neill *et al.* New York: Raven Press.

Watson, S. & Abbott, A. (1991). Muscarinic acetylcholine receptors. *Trends in Pharmacological Sciences* (January 1991 Suppl.) p. 19.

Webley, G. E., Luck, M. R. & Hearn, J. P. (1988). Stimulation of progesterone secretion by cultured human granulosa cells with melatonin and catecholamines. *Journal of Reproduction and Fertility* **84,** 669–677.

Zacchetti, D., Celmenti, E., Fasolata, C., Lorenzon, P., Zottini, M., Grohovaz, F., Fumagalli, G., Pozzan, T. & Meldolesi, J. (1991). Intracellular Ca^{2+} pools in PC 12 cells. A unique, rapidly exchanging pool is sensitive to both inositol 1,4,5-triphosphate and caffeine-ryanodine. *Journal of Biological Chemistry* **266,** 20152–20158.