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Intracellular free calcium related to apoptotic cell death in quail granulosa cell sheets kept in serum-free culture

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Abstract

The relationship between apoptosis and resting intracellular free calcium ($[Ca^{2+}]_i$) was studied in serum-free cultures of granulosa cell sheets isolated from preovulatory quail follicles. Apoptosis was detected by acridine orange, *in situ* end-labeling of fragmented DNA and electron microscopy. $[Ca^{2+}]_i$ was measured using fura-2. $[Ca^{2+}]_i$ averaged 525 mM in freshly isolated sheets. In 24 h cultures no apoptosis was detected but $[Ca^{2+}]_i$ became very dispersed, 20% of the sheets showing values above 1000 nM. At 48 h, apoptosis was obvious and $[Ca^{2+}]_i$ remained dispersed. At 72 h, apoptosis and also the fraction of sheets with high $[Ca^{2+}]_i$ were at their maximum. At 96 h apoptosis was subsiding and $[Ca^{2+}]_i$ normalized. FSH depressed apoptosis and $[Ca^{2+}]_i$ in the 72 h cultures. We conclude that at 24 h apoptosis is intitiated at high $[Ca^{2+}]_i$ foci. At later stages apoptosis is associated with high $[Ca^{2+}]_i$, but it is not clear whether this is cause or consequence.

Keywords: follicular atresia, granulosa cells, apoptosis, intracellular free calcium, fura-2 epifluorescence

Abbreviations: GC, granulosa cell; $[Ca^{2+}]_i$, intracellular free calcium

Introduction

Hormone-dependent tissues deprived from their survival factors represent a model to study the mechanisms of physiological or active cell death. It is now established that atresia of ovarian follicles in mammalian (Hughes and Gorospe, 1991) and avian (Tilly *et al*, 1991) vertebrates is initiated by apoptotic cell death of the granulosa cells (Greenwald and Terranova, 1988).

Apoptotic cell death can be triggered by calcium overload as is the case for accidental cell death (Nicotera *et al*, 1992; Trump and Berezesky, 1995). Moreover cytoplasmic and (or) nuclear ionized calcium increases have been implicated in all phases of apoptosis (Corcoran *et al*, 1994; Kroemer *et al*, 1995; McConkey *et al*, 1994). A $[Ca^{2+}]_i$ rise during the induction phase may activate signal transduction pathways involving phosphatases and protein kinases. During the effector phase $[Ca^{2+}]_i$ has been proposed as a cofactor for the anti-apoptosis oncogene Bcl-2. Finally it has been shown that $[Ca^{2+}]_i$ is a pleiotropic activator of proteases (e.g calpain), phospholipases and nucleases during the degradation phase (Trump and Berezesky, 1995; McConkey *et al*, 1994). Furthermore changes in chromatin structure, chromatin unfolding and gene activation are also calcium-dependent processes (for review see Orrenius and Nicotera, 1994).

Evidence for the role of a $[Ca^{2+}]_i$ comes from experimental work in models of apoptosis using widely differing cell types, including granulosa cells, and is based on the fact that increased $[Ca^{2+}]_i$ can induce internucleosomal DNA cleavage in the nucleus (Cohen and Duke, 1984; Jones *et al*, 1989; Zeleznik *et al*, 1989), that calcium-ionophores, which increase $[Ca^{2+}]_i$ can initiate apoptosis (McConkey *et al*, 1989b; 1991; Ojcius *et al*, 1991; Takei and Endo, 1994), that calcium-chelators inhibit apoptosis (Perotti *et al*, 1990) and that apoptosis is often preceded by an increase of $[Ca^{2+}]_i$ (McConkey *et al*, 1989a; 1990; Martikainen and Isaacs, 1990; Bellomo *et al*, 1992; Escargeuil-Blanc *et al*, 1994). However, the picture does not seem to be so clear cut, as several authors have, based on experimental work with calcium-ionophores, calcium-chelators and calcium-sensitive fluorescent dyes, proposed that apoptosis is rather linked to a decrease of $[Ca^{2+}]_i$ (ionophores-chelators: Baffy *et al*, 1993; calcium-sensitive dyes: Bansal *et al*, 1990; Galli *et al*, 1995).

Several model systems have been used to study the molecular mechanisms of granulosa cell death including both *in vivo* (reviewed in Tsafriri and Braw, 1984) and *in vitro* models. The *in vitro* models are based on culturing either complete antral follicles or dissociated granulosa cells in the absence of hormones and under serum-free conditions (Tilly *et al*, 1992; Tilly and Tilly, 1995; Tilly *et al*, 1995; Chun *et al*, 1994; Luciano *et al*, 1994; Eisenhauer *et al*, 1995). In these models the granulosa cells or intact preovulatory follicles were isolated from ovaries of gonadotropin-primed immature rats. In the present study we have developed an *in vitro* model of granulosa cell death using serum-free culture of sheets of granulosa cells isolated from the largest preovulatory follicle of adult untreated quail, an approach in which the normal tissue architecture is preserved compared to the dissociated granulosa cell preparations. The aim of this work was to study the pattern of resting $[Ca^{2+}]_i$ changes, in relation to the onset and progression of apoptotic degeneration.

Results

Viability and apoptosis (*in vitro*)

Granulosa cell sheets kept in serum-free culture for 24 h appeared as a flat and stretched-out preparation. Careful microscopic observation did reveal a rare apoptotic cell. There was a remarkable increase in number and extent of gap junctions between adjacent cells as compared to freshly isolated granulosa cell sheets, sometimes these gap junctions could reach a length of several micrometers (Figure 1). The granulosa cells in these 24 h cultures typically remained attached to their basement membrane and vitelline membrane. *In situ* end-labeling did not show any evidence for DNA fragmentation while positive controls with DNAse treatment displayed intense staining (Figure 2a, b). Acridine orange staining revealed a normal chromatin configuration (Figure 2c).

Granulosa cell sheets cultured for 48 to 96 h under serum-free conditions differed macroscopically from the 24 h cultures in that these sheets progressively changed from a stretched to a curled and crumpled appearance. Microscopically, the monolayer of granulosa cells sandwiched between their basement membrane and vitelline membrane also progressively disintegrated. At several places the epithelial sheets detached from the membranes. The number of apoptotic cells evaluated in haematoxylin-eosin (H&E) stained sections (Figure 2d) amounted at culture time 24 h to $0.5 \pm 0.1\%$, $4.7 \pm 1.3\%$ SEM at 48 h culture time and reached a maximum at 72 h ($10.5 \pm 1.4\%$ SEM). Surprisingly the number declined afterwards ($7.1 \pm 2.1\%$ SEM) (see Table 1). On the vitally-stained sheets counting of apoptotic cells was not reliable due to the curled and crumpled appearance of the sheets, which hampered focusing (Figure 2e). *In situ* end-labeling confirmed the data obtained on H&E stained sections, moreover a number of nuclei with normal chromatin configuration on H&E stained sections were positive after *in situ* end-labeling (Figure 2f). Electron microscopy at 48, 72 and 96 h revealed that some apoptotic bodies were engulfed by adjacent viable granulosa cells while most were undergoing secondary necrosis as is usually the case in *in vitro* systems. Gap junctions became less abundant and smaller when compared to the 24 h cultures. Cells surviving at 96 h showed normal nuclei and (near) normal structure of mitochondria, endoplasmic reticulum and numerous ribosomes (Figure 3).

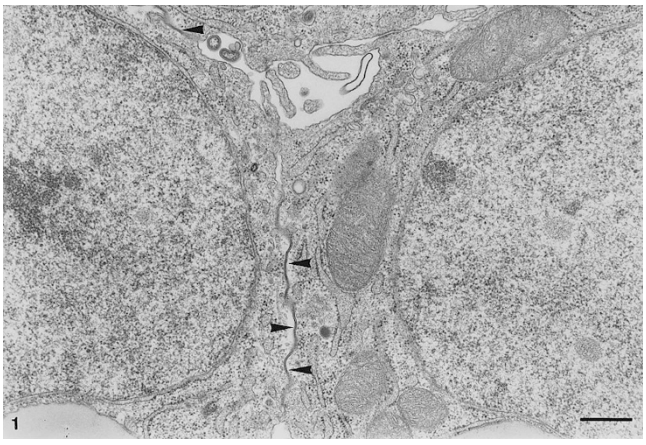

Figure 1 GC-sheet after 24 h culture in serum-free medium. Normal appearance of cytoplasm and nuclei, large gap junctions between neighbouring granulosa cells (arrowheads). Mag: $\times 24\,000$, Bar: 0.5 μm.

In cultures supplemented with FSH (100 ng/ml) the apoptotic process could be markedly reduced. At culture times 48, 72 and 96 h counts on H&E sections resulted in respectively 2.3 ± 0.8 SEM, 3.0 ± 1.1 SEM and $3.9 \pm 1.3\%$ SEM of apoptotic nuclei (see Table 1)

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was measured in acutely isolated granulosa cell sheets and in sheets kept for 24, 48, 72 and 96 h in serum-free culture. Moreover $[Ca^{2+}]_i$ was measured in cell sheets kept for 72 h in serum-free culture medium to which FSH (100 ng/ml) was added. Before the start of the calibration procedure, the preparations were stimulated by superfusion during 4 min with the agonists carbachol (1 – 10 mM), LH (1 IU/ml) and cAMP (1 – 10 mM). In only one fourth of the tested preparations (n=12) a response of $[Ca^{2+}]_i$ could be monitored with carbachol. In contrast the non-specific agent DMSO (1 – 2%) was able to elicit a small response in all sheets where the resting $[Ca^{2+}]_i$ was not close to the saturation level of the calcium probe (Figure 4). Figure 4 demonstrates a typical trace of the ratio signal during stimulation with agonists and during calibration.

Resting $[Ca^{2+}]_i$ averaged 525 ± 75 nM (n=6) in acutely isolated granulosa cell sheets. In the 24 h culture group the range of measured $[Ca^{2+}]_i$ values was much more smeared out, averaging 606 ± 176 nM (n=15). In one sheet the saturation level of fura-2 was reached, corresponding to a $[Ca^{2+}]_i$ level equal to or above 2500 nM. Twenty percent of the sheets showed a $[Ca^{2+}]_i$ level above 1000 nM. A similar pattern was found in the 48 h cultures, where 17% of the sheets displayed a $[Ca^{2+}]_i$ level above 1000 nM. However the distribution of the $[Ca^{2+}]_i$ values in the latter cultures was not as continuous as in the 24 h cultures displaying only low and very high values. This can be due to the smaller size of the experimental group (n=6 versus n=15 at 24 h).

In the 72 h cultures the fraction of sheets with $[Ca^{2+}]_i$ above 1000 nM had risen to 57%. Again, only low and very high values were observed here. At 96 h culture all sheets showed a low $[Ca^{2+}]_i$ value, averaging 89.1 ± 15.6 nM (n=7). From these data (summarized in Figure 5) it appears that the large fraction of sheets with a high $[Ca^{2+}]_i$ value correspond to a culture stage where apoptosis is at its maximum (72 h cultures). In 72 h cultures where apoptosis was inhibited by FSH, no sheets were found with $[Ca^{2+}]_i$ above 1000 nM; $[Ca^{2+}]_i$ averaged to 305 ± 124 nM (n=6) in this group. This value is significantly lower ($p < 0.025$) than the $[Ca^{2+}]_i$ level in the 72 h cultures without FSH i.e. 1340 ± 412 nM (n=7).

Discussion

The present study shows that apoptosis is induced by culturing granulosa cell sheets from adult untreated animals for 48 h under serum-free conditions.

Electron microscopy, *in situ* end-labeling and acridine orange staining demonstrate that GC sheets cultured for up to 24 h under serum-free conditions provide a preparation of viable, non-apoptotic cells. Counts on H&E revealed a percentage of apoptotic nuclei smaller than one. Gap junctions were numerous and large in these cultures while it is known that in freshly isolated avian granulosa they are rare (see Perry *et al*, 1978; D'Herde and Vakaet, 1992); their development in our *in vitro* model may be related to the presence of the basement membrane. Indeed basement membrane components, beside their substantial role in the maintenance and further propagation of granulosa cell differentiation *in vitro*, can stimulate *de novo* gap junction formation in these cells as was demonstrated by morphometrical analysis: the cell membrane occupied by gap junctions was 4–5 times greater in cells grown on basement membrane compared to freshly isolated cells (Amsterdam *et al*, 1989).

Keeping the GC sheets in serum-free culture for a period longer than 24 h elicited manifest apoptosis with a

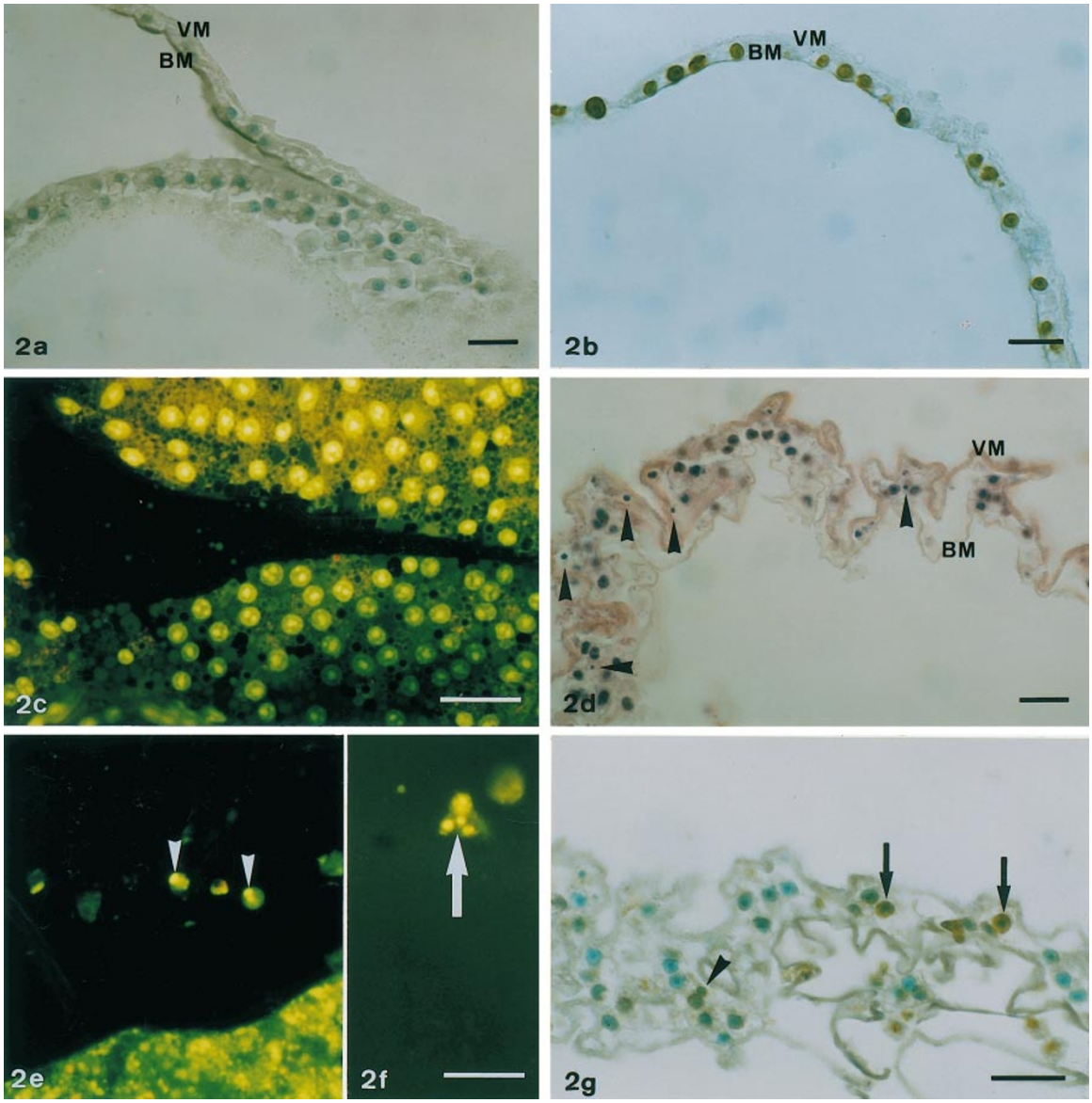

Figure 2 (**a**) GC-sheet after 24 h culture in. *In situ* 3′-end labeling of DNA (brown reactionproduct and methylgreen counterstaining); no evidence of DNA fragmentation. Mag: ×400, Bar: 20 μm. (**b**) Positive control with DNAse treatment. *In situ* end-labeling of DNA fragments; all nuclei are accessible to the incorporating enzyme, indicating that absence of staining in **a** is due to absence of DNA fragmentation. VM: vitelline membrane, BM: basement membrane, Mag: ×500, Bar: 20 μm. (**c**) GC-sheet after 24 h culture. Supravital staining with acridine orange reveals typical appearance of normal quail nuclei characterized by a centrally placed, large nucleolus-associated heterochromatin mass. Mag: ×560, Bar: 20 μm. (**d**) GC-sheet after 72 h culture, H&E stained 3 μm section. Many GC's are detached from the basement membrane (BM); the vitelline membrane (VM) and BM are highly wrinkled. In between normal nuclei are a number of condensed chromatin masses of apoptotic nuclei (arrowheads). Mag: ×400, Bar: 20 μm. (**e–f**) GC-sheet after 72 h culture, acridine orange staining. Condensation of chromatin (arrowheads) and fragmentation of nuclei (arrow) in isolated cells. (**e**) GC-sheet itself, its curled aspect hampers focusing. Mag: ×560, Bar: 20 μm. (**g**) GC-sheet after 72 h culture. *In situ* 3′-end-labeling of DNA fragments (brown reactionproduct and methylgreen counterstaining). Labeling at the periphery of a condensed chromatin mass (arrowhead). Remark also the presence of labeling in nuclei with normal structure (arrows) Mag: ×560, Bar: 20 μm.

Table 1 Frequency of apoptotic nuclei as a function of culture time and expressed as the percentage of total nuclei counted in the same microscopic fields of H&E stained sections (mean+SEM of five and three separate experiments)

	Serum-free cultures *n*=5 (%)	FSH supplemented cultures *n*=3 (%)
24 h	0.5+0.1	0.5+0.3
48 h	4.7+1.3	2.3+0.8
72 h	10.5+1.4	*3.0+1.1
96 h	7.1+2.1	3.9+1.3

*Means significantly different from the data in the serum-free 72 h culture group with $p<0.01$.

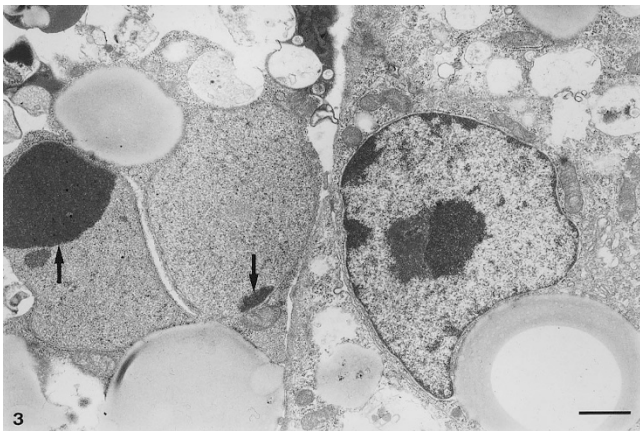

Figure 3 GC-sheet after 96 h culture. Two apoptotic nuclear fragments with marginated condensed chromatin (arrows), adjacent cell has normal nucleus. Although the cytoplasm shows vacuoles, there is near normal morphology of mitochondria, endoplasmic reticulum and numerous ribosomes. Mag: 1 2000, Bar: 1 μm.

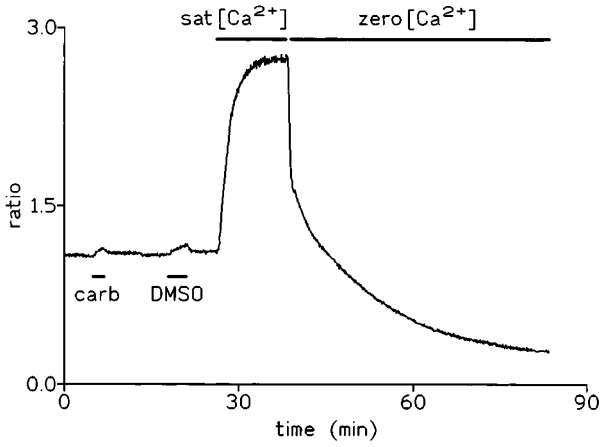

Figure 4 Time course of change of the fura-2 fluorescence ratio signal, measured as a mean signal over a subregion in a granulosa cell sheet, during stimulation with carbachol (carb, 10 mM) or DMSO (1 mM) and during calibration. Calibration involves sequential superfusion of the preparation with a solution that saturates fura-2 (sat. $[Ca^{2+}]$) and with a solution that contains no free calcium (zero $[Ca^{2+}]$).

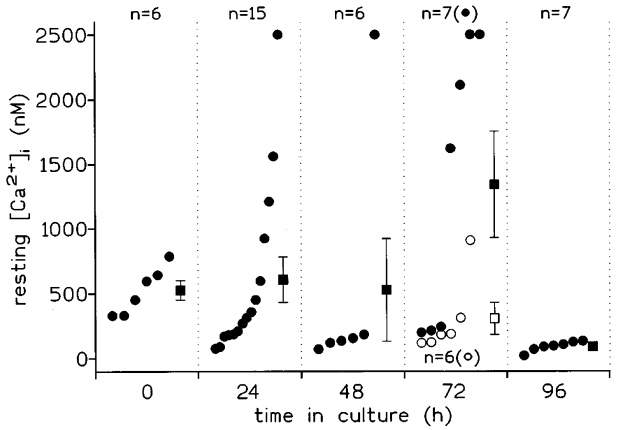

Figure 5 Plot of the resting $[Ca^{2+}]_i$ levels in granulosa cell sheets in function of the culture time. Individual measurements are represented by the filled circles, ordered from low to high values. The mean of each culture group is represented by the filled squares; the wiskers show the SEM. The open circles and squares in the 72 h culture group show measurements with FSH added to the culture medium. $[Ca^{2+}]_i$ values in this group were significantly lower ($p<0.025$) compared to 72 h culture without FSH.

maximum occurring at 72 h. This time sequence differs from the model of apoptosis using dissociated large granulosa cells from immature rat, in which manifest apoptosis already appeared after 24 h of serum-free culture in 50% of the cells (Luciano *et al*, 1994). The apoptosis-free period observed in our study is comparable to *in vivo* situations and may be related to the presence of both basement membrane and intercellular junctions in the GC sheet preparation. The presence of basement membrane can suppress the appearance of apoptosis in mammary epithelial cells (Boudreau *et al*, 1995). Peluso and Pappalardo (1994) have reported that, in their cultures of dissociated granulosa cells, significantly less apoptosis occurred in reaggregated cells connected by gap junctions. Recently the same group (Peluso *et al*, 1996) reported evidences that the anti-apoptotic action of cell contact in reaggregated GC's is not due to the presence of gap junctions, but is mediated by adhesion-type junctions.

Bursch *et al* (1990) calculated that, if the apoptotic process takes 3 h to complete one must only find 2 to 3% of the cells in a tissue to be undergoing apoptosis at any one time to obtain a very substantial cell loss of approximately 25% in 24 h.

If the duration of the apoptotic process in our *in vitro* system can be compared to the latter data from *in vivo* systems than one can calculate that 10.5% apoptotic nuclei at culture stage 72 h (without FSH) will result in a cell loss of over 50% in the next 24 h. We found a lower number of apoptotic nuclei in the 96 h culture (7.1%) which indicates the progressive selection of a subpopulation of GC's which accommodates to serum-free culture and hormone deprivation. For the granulosa of preovulatory hen follicles it has been shown by multiparameter flow cytometry that two subpopulations do exist in a ratio of one to four; they differ

in protein and RNA contents, size, cell cycle characteristics and DNA stainability (Marrone and Crissman, 1987). Interestingly in the mammalian model also (Luciano *et al*, 1994) a fraction of GC's was not triggered by serum-free culture to develop apoptosis.

The presence of apoptosis in the GC sheets extending over several days, while in each cell the process of apoptotic degeneration takes only 3 h, points to asynchronous triggering throughout the sheet, and thus is comparable to what is observed in *in vivo* models: for example apoptosis induced in the ventral prostate gland by castration (Colombel and Buttyan, 1995). Including FSH in the culture medium inhibited apoptosis in the present model system as expected from its *in vivo* effects (Billig *et al*, 1994). Inhibition of spontaneous apoptosis by FSH was demonstrated in cultured rat follicles (Tilly and Tilly, 1995), but not yet in model systems containing only granulosa cells.

From the agonists tested, only carbachol and DMSO resulted in a $[Ca^{2+}]_i$ response. In dissociated single chicken granulosa cells $[Ca^{2+}]_i$ transients could be evoked by LH and cAMP as well (Hertelendy *et al*, 1989; Morley *et al*, 1992; Morley and Whitfield, 1994). A possible explanation for the lack of response in our experiments can be that only a fraction of the cells responds to these agonists, which is presumably not detected in average measurements over a hundred cells as performed in the present experiments. Another explanation is that the appropriate receptors are lost during culture.

Calcium measurements in acutely isolated granulosa cell sheets showed a resting $[Ca^{2+}]_i$ value of 525 nM. This is rather high when compared to the 100 nM reported by Morley *et al* (1992) for dissociated and cultured GC's of chicken F1 follicles. It is slightly above the 355 nM observed in freshly dissociated GC's of chicken F1 follicles (Hertelendy *et al*, 1989). Keeping the granulosa cell sheets for 24 h in culture elicited in one fifth of the sheets high $[Ca^{2+}]_i$ values. At this culture stage there was no manifest apoptosis, suggesting that the increased $[Ca^{2+}]_i$ is involved in triggering apoptosis that is to appear 24 h later. Luciano *et al* (1994) have also reported hat apoptosis is preceded by a transient increase of $[Ca^{2+}]_i$ using a model of serum-free culture of dissociated rat granulosa cells. The $[Ca^{2+}]_i$ levels at the 24 h culture stage were equal to or higher than the $[Ca^{2+}]_i$ levels that have been reported to precede apoptosis in other model systems (McConkey *et al*, 1989a: 320–811 nM; McConkey *et al*, 1990: 457 nM; Bellomo *et al*, 1992: 600 nM).

$[Ca^{2+}]_i$ at culture stage 72 h was significantly lower when FSH was included in the culture medium. As FSH also inhibited apoptosis, this suggests a correlation between apoptosis and $[Ca^{2+}]_i$. Finally at 96 h of culture $[Ca^{2+}]_i$ attained a normal value of approximately 90 nM. At this culture stage the apoptotic process was subsiding, suggesting that the surviving small fraction of cells is resistant to the serum-free culture conditions and hence do not undergo apoptosis.

Taken together the present data suggest that apoptosis is initiated at high calcium foci within the granulosa cell layer at the 24 h serum-free culture stage. The high $[Ca^{2+}]_i$ at 48 and 72 h can be interpreted as either initiating apoptosis or else as a consequence of the apoptotic process. Further experimental work aimed at correlating the calcium signal with markers of apoptosis at the level of individual cells will be needed to gain deeper insight in the relationship between $[Ca^{2+}]_i$ and apoptosis in the presently used model.

Materials and Methods

Isolation and culture of GC sheets

GC sheets were prepared from ovarian follicles of adult regularly laying Japanese quail (Coturnix coturnix japonica). The animals were reared under continuous artificial illumination, with food and water ad libitum. The monolayered granulosa layer of the largest preovulatory follicle (F1) was isolated from the surrounding the cal covering in Krebs-Ringer equilibrated with a gas mixture of 80% O_2 and 20% CO_2, according to the technique described by Gilbert *et al* (1977). This method provides large sheets of vital GC's, sandwiched between their basement membrane and vitelline membrane. Granulosa cell sheets of circa 4 mm^2 were maintained in 35 mm culture dishes under serum-free conditions for up to 96 h in humidified room air at 37°C. The culture medium was M199 (Sigma, cat. no. M-039, Bornem, Belgium) supplemented with 0.1% bovine serum albumin (fraction V, Sigma, cat. no. A-4503, Bornem, Belgium), 6.0 g/l HEPES, 50 U/ml penicillin and 50 mg/ml streptomycin at pH 7.4. In order to inhibit the apoptotic process (Tilly and Tilly, 1995) culture medium was supplemented with 100 ng/ml sheep pituitary FSH (NIH-FSH-S17 U/mg Sigma, cat.no. F-4520) in control cultures.

Electron microscopy

The granulosa cell sheets were fixed by immersion for 3 h in 2% glutaraldehyde and 1 mM $CaCl_2$ buffered with 100 mM Na-cacodylate (pH 7.4). After a rinse in 100 mM Na-cacodylate containing 7.5% sucrose, the sheets were osmicated in 2% OsO_4 in 100 mM Na-cacodylate and embedded in LX medium (Ladd, Burlington, VT, USA). Semithin sections were contrasted with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate.

In situ end-labeling (ISEL) of DNA fragments

In situ end-labeling was performed on paraffin sections of the GC sheets. DNA fragments were 3′-end-labeled by use of the terminal transferase reaction (ApopTag S7100-kit, Oncor, Gaithersburg, MD, USA) according to a procedure described previously (D'Herde *et al*, 1994). Positive controls were first treated with DNase I to introduce DNA breaks in all nuclei.

Staining of apoptotic nuclei

Apoptotic nuclei were visualized by vital staining with acridine orange. A dye-mix working solution of 100 μg/ml acridine orange (BDH, Poole, UK) was prepared in normal buffer solution (NBS). The composition of NBS was as follows: 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, 2.6 mM dextrose and 10 mM HEPES, pH 7.4. The GC sheets were superfused during 5 min with NBS

containing 4 μg/ml acridine orange. After rinsing with NBS the GC sheets were examined with a $\times 40$ objective using a filter combination suitable for observing fluorescein.

Quantification of apoptotic nuclei

Apoptotic cells were identified by their characteristic morphological features as seen on 3 μm H&E stained sections. Apoptosis was scored in ten $100\times$ randomly selected microscopic fields of an individual section and expressed as the number of apoptotic cells per number of total nuclei counted in the same microscopic fields. Depending on the culture time a total number of 500 to 750 nuclei were counted per GC sheet. Small groups of apoptotic bodies were counted as remnants of one apoptotic cell.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using the calcium-sensitive fluorescent indicator fura-2 in combination with epifluorescence microscopy (Nikon Diaphot microscope, $\times 40$ oil immmersion objective). GC sheets (cultured for 24 or 48 h) were loaded with fura-2 by incubating them in NBS containing 5 μM fura-2 acetoxymethyl-ester (fura-2-AM, Molecular Probes, cat. no. F-1201, Eugene, OR, USA) and 0.01% (w/v) pluronic during 90 min at room temperature. GC sheets cultured for longer periods (72 and 96 h) generally needed longer incubation times (120 min) to obtain a measurable fluorescence signal. The loading solution also contained 2.5 mM probenecid (Sigma, cat. no. P-8761, Bornem, Belgium) to prevent dye secretion (Di Virgilio *et al*, 1988). After loading, the GC sheets were rinsed twice and further incubated in NBS at 37°C during 1–2 h to ensure de-esterification. GC sheets were then ready for experiments. GC sheets were placed, one at a time, in the recording chamber and mechanically fixed using an overlaying grid of nylon threads attached to a metal ring. The recording chamber consisted of a Petri dish with a coverslip bottom. The GC sheet was submerged in a droplet of NBS; fresh NBS equilibrated with 100% O_2 and at room temperature entered the droplet at a rate of 1 ml/min via a pipette positioned close to the sheet. All reagents (e.g. agonists to stimulate the preparation, calibration solutions) were added via this superfusion pipette. Superfluous solution was drained via a second pipette inserted at the surface of the droplet and connected to a water jet pump. The volume of the droplet was kept constant at approximately 100 μl.

$[Ca^{2+}]_i$ was determined from the fluorescence ratio resulting from excitation with light at 340 and 380 nm. A detailed description of the setup can be found in Leybaert *et al* (1993). Once a stable baseline ratio signal was established, the preparation was stimulated by superfusion with the agonists carbachol (Sigma, cat. no. C-4382, Bornem, Belgium), 8-Br-cAMP (Sigma, cat. no. B-7880, Bornem, Belgium), luteinizing hormone (LH) (Human choriongonadotrophin, Pregnyl (^{R}), ampoules 1500 IU, Organon, Belgium) or with the non-specific agonist dimethyl-sulfoxide (DMSO) (DMSO for gas chromatography, Merck, Darmstadt, Germany). After this stimulation protocol, an *in situ* calibration procedure was carried out. This involved superfusion of the preparation with a fura-2 saturating solution (containing 145 mM KCl, 10 mM $CaCl_2$, 10 mM HEPES at pH 7.4) and consecutively with a calcium-free solution (containing 145 mM KCl, 10 mM EGTA, 10 mM HEPES at pH 7.4) both in combination with 20 μM of the calcium ionophore 4-Br-A-23187 (Molecular Probes, cat. no. B-1404, Eugene, OR, USA). This procedure allowed to determine R_{max}, R_{min} and β, required to convert the fluorescence ratio to $[Ca^{2+}+]_i$ using the formula described by Grynkiewicz *et al* (1985). A dissociation constant of 224 nM was used for the binding of fura-2 with calcium. The dynamic range of the ratio measurements (R_{max}/R_{min}), which gives an idea of the degree of de-esterification of the calcium probe, was not different between the different culture stages; it averaged 9.89 ± 1.74 (mean$\pm$SEM) over all measurements (n=47). Comparison of the fluorescence level between fura-2 loaded GC sheets and solutions with known fura-2 (pentapotassium salt) concentrations suggest an intracellular fura-2 concentration below 50 μM.

Statistical analysis

Statistical significance was tested using the Mann-Whitney U-test, a non-parametric test for unpaired observations. A p value less than 0.05 was considered as statistically significant (one-tailed test).

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