Hormonal regulation of apoptosis in rabbit granulosa cells *in vitro*: evaluation by flow cytometric detection of plasma membrane phosphatidylserine externalization

G. Maillet, E. Bréard, A. Benhaïm, P. Leymarie and C. Féral

Laboratoire de Biochimie, UPRES EA 2608, CHU Côte de Nacre, Université de Caen, 14032 Caen Cedex, France

Annexin V and propidium iodide bivariate analysis and the TUNEL method were used to quantify hormonal regulation of apoptosis in rabbit granulosa cells from preovulatory follicles *in vitro*. The aim of this study was to analyse comparatively the effects of gonadotrophins and their second messenger in the regulation of granulosa cell apoptosis in (i) cultured isolated granulosa cells and (ii) granulosa cells scraped from cultured follicles. The results showed that increasing doses of FSH had no effect on apoptosis of cultured isolated cells but caused a decrease in the number of apoptotic granulosa cells from preovulatory follicles cultured in serum-free conditions. Unlike FSH, addition of hCG did not modify apoptosis of

granulosa cells significantly. In contrast, dibutyryl cAMP had an apoptotic effect in the two cellular models in the presence of serum. Moreover, a biphasic effect of dibutyryl cAMP in isolated granulosa cells was observed with an increase in the incorporation of [³H]thymidine into DNA at the lowest dose and an increase in apoptotic cell death at the highest dose. It was concluded that, in rabbits: (i) FSH requires follicle integrity to exert its anti-apoptotic effect in granulosa cells; (ii) dibutyryl cAMP induces a dose-dependent apoptotic effect in granulosa cells cultured alone or obtained from cultured preovulatory follicles; and (iii) cAMP signals induce opposite effects on growth and apoptosis in granulosa cells.

Introduction

Apoptotic cell death of granulosa cells is the underlying event associated with initiation of follicular atresia (Hughes and Gorospe, 1991; Tilly *et al.*, 1991, 1992). Gonadotrophins play a major role in the development of ovarian follicles and, thus, FSH and LH are likely to be survival factors in follicular apoptosis. In most studies, the ability of gonadotrophins to suppress apoptosis has been tested *in vivo* (Billig *et al.*, 1994) or *in vitro* in an intact follicle culture system (Chun *et al.*, 1994, 1996; Kaipia *et al.*, 1996). When tested in isolated granulosa cells, FSH was ineffective in rats (Aharoni *et al.*, 1995), whereas it attenuated apoptosis in pigs (Guthrie *et al.*, 1998) and cows (Yang and Rajamahendran, 2000; Hu *et al.*, 2001).

The effects of gonadotrophins are exerted via an increase in intracellular cAMP. The effect of the cAMP analogue, dibutyryl cAMP (dbcAMP), on follicular apoptosis is controversial. Indeed, dbcAMP was described as apoptotic in isolated granulosa cells from preovulatory rat follicles (Aharoni *et al.*, 1995) but as a survival factor in cultured early antral rat follicles (Chun *et al.*, 1996).

No study has been performed using both isolated granulosa cells and cultured follicles to study the role of

paracrine interactions between follicular cells in the action of gonadotrophins against apoptosis, as was suggested by Hsueh *et al.* (1996).

Apoptosis is characterized by internucleosomal DNA cleavage in association with morphological changes including membrane blebbing, nuclear condensation or fragmentation and formation of apoptotic bodies. In addition, cells induced to undergo apoptosis show a loss of phospholipid asymmetry in the plasma membrane, which results in the exposure of phosphatidylserine on the outer leaflet. This phosphatidylserine exposure can be used to detect apoptosis in vital, non-fixed cells by allowing fluorescein isothiocyanate (FITC)-labelled annexin V to bind to phosphatidylserine.

On the basis of the above considerations, the aim of the present study was to determine whether gonadotrophins can modulate apoptosis in isolated rabbit granulosa cells or in granulosa cells obtained from cultured preovulatory rabbit follicles. The role of dbcAMP in the regulation of apoptotic death of granulosa cells was also studied in parallel with [³H]thymidine incorporation and steroid production. Apoptosis was evaluated in granulosa cells using the propidium iodide and annexin V bivariate analysis proposed by Vermes *et al.* (1995). This method was used for the detection of the early stage of apoptosis and, in contrast to the TUNEL method, allowed rapid analysis of data.

Email: maillet.geraldine@caramail.com

Materials and Methods

Animals and treatments

HY female white rabbits, aged 10–12 weeks (Elevage Gastebled, Hottot Les Bagues), were housed individually for about 2 weeks on a 14 h light (from 06:00 h to 20:00 h) and 10 h dark schedule with standard rabbit food and water available *ad libitum*. Animals were bred under standard conditions according to the instructions of Ministère de l'Agriculture et de la Pêche-Service Santé Animale (France).

The development of numerous preovulatory follicles was induced in these rabbits by i.m. injection of 200 iu eCG (Chrono-Gest; Intervet, Paris) daily for 2 days. The animals were killed by intracardiac injection of 2 ml of a narcotic (Embutramide T61; Distrivet, Paris) 4 days after the first injection of eCG.

The ovaries were excized and placed in minimum essential medium (MEM; Eurobio, Les Ulis) containing 20 mmol Hepes l^{-1} (Sigma, St Quentin Fallavier), 50 iu penicillin ml^{-1} (Sigma), 50 μg streptomycin ml^{-1} (Eurobio) and 0.1% (w/v) BSA (Sigma). The follicles were dissected out (those containing clotted blood were discarded) and cleared of interstitial tissue under a stereomicroscope.

Granulosa cell preparation and culture protocols

Dispersed granulosa cells were obtained as described by Féral *et al.* (1995). In brief, follicles were cut in half in MEM–0.1% (w/v) BSA containing 6.8 mmol EGTA l^{-1} (Sigma) and incubated for 10 min. Released granulosa cells and follicles were centrifuged together at 300 *g* for 10 min and resuspended in MEM–0.1% (w/v) BSA containing 0.5 mmol sucrose l^{-1} (Sigma) and 1.8 mmol EGTA l^{-1} for 5 min before centrifugation again at 300 *g* for 10 min. Granulosa cells were gently scraped away from the theca with a microspatula under a dissecting microscope. The dispersed granulosa cells were pelleted by centrifugation at 300 *g* for 10 min, subjected to mechanical dissociation, washed twice in MEM–0.1% (w/v) BSA and resuspended in serum-free or serum-containing medium with or without gonadotrophins.

After 72 h of culture, the media were collected and stored at -20°C until the steroid assays were performed and the cells were treated for apoptosis analysis.

Follicle culture

Preovulatory follicles were dissected from ovaries as described above. Six follicles per treatment group were placed in Falcon 24-well tissue culture plates containing 1 ml culture medium in the absence or presence of gonadotrophins with or without 5% fetal calf serum (FCS) and incubated for 72 h. After culture, the follicles of each treatment group were washed in MEM–0.1% (w/v) BSA and granulosa cells were obtained after treatment with EGTA–sucrose and scraping as described above. The analysis of phosphatidylserine exposure was carried out in the same way as for cultured isolated granulosa cells.

Measurement of phosphatidylserine exposure of granulosa cells by flow cytometry

For analysis of phosphatidylserine exposure, 3×10^5 cells ml^{-1} were cultured in the presence or absence of experimental treatments in Falcon 24-well tissue culture plates for 72 h. Adherent cells were washed twice with warm PBS without Ca^{2+} or Mg^{2+} and were treated for 5 min at 37°C with PBS containing 0.5 g trypsin l^{-1} and 0.2 g EDTA l^{-1} (Eurobio). Trypsin activity was stopped by the addition of medium supplemented with 5% (v/v) FCS.

At the end of the culture period, apoptotic granulosa cells were quantified according to the manufacturer's instructions for annexin V–FITC kit (Boehringer-Ingelheim kit; supplied by Coger, Paris). After two washes with binding buffer, annexin V–FITC (3 μg ml^{-1}) and propidium iodide (400 ng ml^{-1}) were added to the cell suspension (3 $\times 10^5$ cells per 500 μl) and incubated for 10 min in the dark. Granulosa cells were analysed during the hour after incubation in a fluorescence-activated cell sorter (FACScalibur; Becton-Dickinson, Sunnyvale, CA) using a 15 mW argon laser emitting light at 488 nm. The following settings were used for bicolour analysis: 475 V on photomultiplier tubes for both FL1 (FITC) and FL2 (propidium iodide). Compensation for FL1–%FL2 and FL2–%FL1 was 1.0 and 32.5%, respectively. Data analysis was performed with Cell Quest software (Becton Dickinson). Ten thousand cells were analysed in each treatment group. In each experiment, quadrants were fixed using the dot plot of granulosa cells cultured in serum-containing medium as shown (Fig. 1). The diagram of granulosa cells obtained from culture in the presence of serum was considered as a control. The incidence of apoptosis in the control is stated in each figure legend. The results of treatments with gonadotrophins and dbcAMP were expressed as an apoptotic cell percentage of the control.

Measurement of DNA fragmentation by TUNEL method

For the study of DNA fragmentation by terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labelling (TUNEL) method, 2×10^5 cells ml^{-1} were grown onto a round coverslip placed in the bottom of each well of Falcon 24-well tissue culture plates. Cells were prepared according to the manufacturer's indications for the TdT-FragEL™ kit (Amersham, Orsay).

In brief, at the end of culture, cells on coverslips were fixed with 3% (w/v) paraformaldehyde (Merck, Fontenay sous Bois) for 30 min at room temperature, washed intensively with TBS and permeabilized for 5 min with proteinase K (20 μg ml^{-1}) followed by washing with TBS. DNA fragmentation in cultured cells was assessed using the TdT-FragEL™ kit. Coverslips were counterstained with methyl green and mounted using DePeX mounting medium.

A negative control was obtained by omitting the terminal deoxynucleotidyl transferase (TdT) enzyme in the reaction mixture during the labelling step.

Cells with labelled nuclei and total cells were counted in

Fig. 1. Dot plots of annexin V fluorescein isothiocyanate (FITC)–propidium iodide two-parameter flow cytometry of rabbit granulosa cells cultured (a) with fetal calf serum (FCS) (considered as control) and (b) in serum-free conditions. The lower left quadrant contains cells considered as healthy (annexin–/propidium iodide–). The lower right quadrant shows apoptotic cells (annexin+/propidium iodide–) and the upper quadrants represent necrotic and secondary necrotic cells (propidium iodide+). Fluorescence intensities are expressed in arbitrary units.

five random fields scoring 500–800 cells of each treated culture to quantify DNA fragmentation. The percentage of apoptotic nuclei was estimated independently by two people in three independent experiments. Data are expressed as mean $\pm$ SEM.

Measurement of [^3H]thymidine incorporation into DNA

Granulosa cells (10^5 cells per 500 μl) were cultured for 72 h in Falcon 48-well tissue culture plates in serum-containing medium and treated with increasing doses of dbcAMP (Sigma) during the last 48 h. 1 μCi [^3H]thymidine ml^{-1} (25 Ci mmol^{-1}) (Amersham) was added to the culture medium for the final 24 h. The amount of [^3H]thymidine incorporated into DNA was determined as described by Benhaïm *et al.* (1995). The amount of [^3H]thymidine incorporation into DNA was expressed in c.p.m. per 10^5 cells.

Progesterone and oestradiol production

The concentrations of progesterone and oestradiol in culture media were measured by radioimmunoassay using specific antibodies (BioSys, Compiègne). Sensitivity was 3 pg per tube for oestradiol and 5 pg per tube for progesterone.

Statistical analysis

Data regarding the production of steroids, the incorporation of [^3H]thymidine and the percentage of apoptotic cells were expressed as mean $\pm$ SEM of at least three experiments. In each experiment, the different treatments were performed on cells from two rabbits. Data regarding the percentage of

apoptotic cells obtained with the TUNEL method and annexin V binding analysis needed an arcsin transformation to obtain a normal distribution. Differences between treatment groups (raw data for the production of steroids and the incorporation of [^3H]thymidine or transformed data for the percentage of apoptotic cells) were assessed by a two-way ANOVA followed by Fisher's test and considered significant at $P < 0.05$.

Results

Apoptosis in cultured granulosa cells

The study of variation in the number of apoptotic cells in cultured granulosa cells was performed at 24, 48 and 72 h in the presence or absence of 5% serum. The analysis of phosphatidylserine exposure showed that the percentage of apoptotic granulosa cells increased significantly in serum-free conditions compared with culture in the presence of serum at all the times tested (24–72 h of culture; Fig. 2). A time-dependent decrease in the percentage of apoptotic cells was observed. In each case, this decrease was significant at 72 h of culture compared with 24 h ($P < 0.05$) and, thus, further experiments were performed after 72 h of culture.

Granulosa cell apoptosis as shown by analysis of phosphatidylserine exposure was confirmed by the TUNEL method (Table 1). In the presence of serum for 72 h, $29 \pm 3\%$ of cells labelled for fragmented DNA; this percentage was increased 2.6-fold in serum-free conditions ($P < 0.001$).

Fig. 2. Time-dependent effect of culture with (□) or without (■) fetal calf serum (FCS) on percentage of apoptotic rabbit granulosa cells. Data are mean $\pm$ SEM of three experiments performed in triplicate. ^{a-d}Values with different superscripts are significantly different ($P < 0.05$).

Effect of gonadotrophins on apoptosis in isolated cultured granulosa cells

Isolated granulosa cells were cultured for 72 h in the presence or absence of serum. The effect of increasing doses of FSH (NIDDK-o-FSH20; 4453 iu mg^{-1}) and hCG (LH activity) (Organon, Serfontaine) on apoptosis of granulosa cells was evaluated in each culture condition.

In the presence of serum, the addition of FSH at concentrations ranging from 5 ng ml^{-1} to 100 ng ml^{-1} did not affect the percentage of apoptotic granulosa cells significantly. The absence of serum in culture medium induced a 2.3-fold increase in the percentage of apoptotic cells above control in serum conditions. In this serum-free condition, the percentage of apoptotic granulosa cells was not affected by treatment with increasing doses of FSH (Fig. 3a).

The addition of increasing doses of hCG ranging from 10 miu ml^{-1} to 100 miu ml^{-1} in culture medium did not significantly affect the percentage of apoptotic granulosa cells in either the presence or absence of serum (Fig. 4a).

Granulosa cells cultured in serum-containing medium responded to addition of gonadotrophins in terms of steroidogenesis. Addition of FSH (100 ng ml^{-1}) and hCG (100 miu ml^{-1}) increased progesterone production 20- and 14-fold, respectively (data not shown).

Effect of gonadotrophins on apoptosis in granulosa cells from cultured preovulatory follicles

Preovulatory follicles were cultured for 72 h in the presence or absence of serum with or without gonadotrophins to assess further the role of gonadotrophins in modulating granulosa cell apoptosis. Granulosa cells were scraped from these follicles and analysed for apoptosis.

In the presence of serum, addition of increasing doses of FSH did not significantly affect the percentage of apoptotic granulosa cells removed from cultured preovulatory

follicles (Fig. 3b). The absence of serum in culture medium induced a two-fold increase in the percentage of apoptotic cells compared with the control in serum conditions when granulosa cells were removed from cultured preovulatory follicles. In this serum-free culture, addition of FSH induced a dose-dependent decrease in the percentage of apoptotic granulosa cells.

The treatment of cultured preovulatory follicles with increasing doses of hCG did not significantly affect the percentage of apoptotic granulosa cells in either serum-containing medium or in serum-free conditions (Fig. 4b).

Induction of apoptosis by increasing doses of dbcAMP in cultured granulosa cells

Cells were cultured with or without serum in the presence of increasing concentrations (0.1–5.0 mmol l^{-1}) of dbcAMP to test the effects of the gonadotrophin second messenger cAMP on granulosa cell apoptosis. When granulosa cells were cultured in the presence of serum, the percentage of apoptotic cells was increased by dbcAMP in a dose-dependent way: 5 $\text{mmol dbcAMP l}^{-1}$ induced a 70% increase in the number of apoptotic cells compared with the control (Fig. 5a). The absence of change in the percentage of propidium iodide-unlabelled cells (cells without a damaged membrane) with increasing doses of dbcAMP ($98 \pm 1\%$ with 0.1 $\text{mmol dbcAMP l}^{-1}$ and $95 \pm 1\%$ with 5 $\text{mmol dbcAMP l}^{-1}$; $n = 3$) excluded a cytotoxic effect of high doses of dbcAMP. These results obtained with the annexin V binding assay were confirmed by TUNEL analysis; treatment with dbcAMP induced a dose-dependent increase in the percentage of apoptotic cells compared with culture in the presence of serum only (Table 1).

The effects of dbcAMP on apoptosis in granulosa cells cultured in serum-containing media were compared with [^3H]thymidine incorporation into DNA (Fig. 6). The incorporation of [^3H]thymidine was increased by 130% at 0.1 $\text{mmol dbcAMP l}^{-1}$ compared with the control ($P < 0.001$), whereas 2.5 $\text{mmol dbcAMP l}^{-1}$ reduced the incorporation of [^3H]thymidine by 30% ($P < 0.001$).

Progesterone and oestradiol were also stimulated by dbcAMP in a dose-dependent manner (Fig. 7).

In the absence of serum, increasing doses of dbcAMP had no significant effect on the percentage of apoptotic granulosa cells, except for the high dose of 5 $\text{mmol dbcAMP l}^{-1}$ (Fig. 5a).

Effect of increasing doses of dbcAMP on apoptosis in granulosa cells from cultured preovulatory follicles

As for gonadotrophins, the effect of dbcAMP on the percentage of apoptotic granulosa cells was tested on preovulatory follicles cultured in the presence or absence of serum. When granulosa cells were scraped from follicles cultured in the presence of serum (Fig. 5b), dbcAMP induced an increase in the percentage of apoptotic granulosa cells in a dose-dependent way, reaching a three-fold increase at 5 $\text{mmol dbcAMP l}^{-1}$.

Table 1. Analysis of the effects of fetal calf serum (FCS) deprivation and increasing doses of dibutyryl cAMP (dbcAMP) on rabbit granulosa cell apoptosis detected by the TUNEL method

	+ FCS (+ dbcAMP (mmol l ⁻¹))				- FCS
	0	0.1	2.5	5.0	
Percentage of apoptotic nuclei (n = 3) ^a	29 ± 3	32 ± 4	47 ± 3**	55 ± 3***	77 ± 7***

^aData are expressed as the mean percentage ± SEM obtained by scoring about 500–800 total cell nuclei in five different fields for each treatment.

Values are mean ± SEM of three experiments.

P* < 0.01 and *P* < 0.001 compared with serum only value (+ FCS).

Fig. 3. Effect of increasing doses of FSH on apoptosis in rabbit (a) isolated granulosa cells and (b) granulosa cells scraped from preovulatory follicles, both cultured for 72 h in the presence (□) or absence (■) of fetal calf serum (FCS). (a) Data are mean ± SEM of three experiments performed in triplicate. (b) Data are mean ± SEM of five experiments. In (a), control = 23.6 ± 5.8% apoptotic cells and in (b), control = 6.7 ± 0.9% apoptotic cells. ^{a-d}Values in a panel with different superscripts are significantly different (*P* < 0.05).

When follicles were cultured in the absence of serum, a three-fold increase in the percentage of apoptotic granulosa cells was observed. This percentage was not changed significantly by treatment with increasing concentrations of dbcAMP.

Fig. 4. Effect of increasing doses of hCG on apoptosis in rabbit (a) isolated granulosa cells and (b) granulosa cells scraped from preovulatory follicles, both cultured for 72 h in the presence (□) or absence (■) of fetal calf serum (FCS). (a) Data are mean ± SEM of three experiments performed in triplicate. (b) Data are mean ± SEM of five experiments. In (a), control = 20.4 ± 4.1% apoptotic cells and in (b), control = 6.1 ± 1.0% apoptotic cells. ^{a-b}Values in a panel with different superscripts are significantly different (*P* < 0.05).

In this study, the effect of gonadotrophins on the degree of apoptosis in isolated rabbit granulosa cells and in granulosa cells obtained from cultured preovulatory rabbit follicles in

Discussion

Fig. 5. Effect of increasing doses of dibutyryl cAMP (dbcAMP) on apoptosis in rabbit (a) isolated granulosa cells and (b) granulosa cells scraped from preovulatory follicles, both cultured for 72 h in the presence (□) or absence (■) of fetal calf serum (FCS). (a) Data are mean $\pm$ SEM of three experiments performed in duplicate. (b) Data are mean $\pm$ SEM of five experiments. In (a), control = $24 \pm 2\%$ apoptotic cells and in (b), control = $3.2 \pm 0.4\%$ apoptotic cells. ^{a-d}Values with different superscripts are significantly different ($P < 0.05$).

the presence or absence of serum was compared in the same experiment. Although the increase in the percentage of apoptotic granulosa cells caused by serum deprivation was equivalent in isolated cells and in cells from cultured follicles (about 2.3-fold compared with serum conditions), the incidence of apoptosis in serum-containing medium was greater when granulosa cells were cultured alone than when they were obtained from cultured preovulatory follicles (23% versus 6% apoptotic granulosa cells, respectively). This finding indicates that there may be a protective effect of preovulatory follicle integrity on apoptosis of granulosa cells. Indeed, cell-cell adhesion sites and particularly cell adhesion molecules have been implicated in survival of human granulosa cells (Makrigiannakis *et al.*, 1999), indicating that the dispersion of granulosa cells for culture might induce apoptosis. Moreover, the presence of

Fig. 6. Effect of increasing doses of dibutyryl cAMP (dbcAMP) on the incorporation of [³H]thymidine into DNA in rabbit granulosa cells after 72 h of culture in the presence of fetal calf serum (FCS). Data are mean $\pm$ SEM of three experiments performed in triplicate. ** $P < 0.01$ and *** $P < 0.001$ compared with the control value.

anti-apoptotic factors in follicular fluid resulting from interactions between granulosa and theca cells could also explain the difference in the percentage of apoptotic granulosa cells between the two culture models. The necessity of co-operation between follicular cells against apoptosis was suggested by Hsueh *et al.* (1996). Indeed, in rats, FSH and insulin-like growth factor I (IGF-I) inhibited apoptosis in cultured preovulatory follicles (Chun *et al.*, 1994), but were ineffective in isolated cultured granulosa cells removed from preovulatory follicles (Tilly *et al.*, 1992; Aharoni *et al.*, 1995).

In our study, no anti-apoptotic effect of increasing doses of FSH in isolated rabbit granulosa cells was observed whatever the conditions of culture. Although FSH has been demonstrated to be a major survival factor in the ovary *in vivo* (Billig *et al.*, 1994), its effect on apoptosis of isolated granulosa cells *in vitro* is controversial. In serum-free conditions, FSH was ineffective in isolated cultured granulosa cells removed from rat preovulatory follicles (Aharoni *et al.*, 1995) and attenuated apoptosis in cultured pig (Guthrie *et al.*, 1998) and bovine (Yang and Rajamahendran, 2000) granulosa cells from small and medium-sized follicles. The anti-apoptotic effect of FSH appears to depend on the differentiation status of isolated granulosa cells. In preovulatory follicles cultured in serum-containing medium, the percentage of apoptotic granulosa cells was low (about 6%) and increasing doses of FSH or hCG did not decrease this percentage. The protective effect of FSH against apoptosis was only observed when rabbit granulosa cells were isolated after the culture of intact follicles in serum-free conditions and this effect was dose-dependent. This result was in agreement with the results of Chun *et al.* (1994) in rat preovulatory follicles *in vitro*.

As in the presence of FSH, no significant effect of hCG on apoptosis was observed in cultured isolated granulosa cells. This finding is in agreement with the results of Aharoni *et al.*

(1995). In contrast, Dharmarajan *et al.* (1994) reported an anti-apoptotic effect of hCG on apoptosis induced by the absence of serum during 2 h culture of rabbit luteal cells. The different status of ovarian cells used could explain this discrepancy. In contrast to FSH, no significant anti-apoptotic effect of hCG was observed in granulosa cells analysed after 72 h of culture of intact follicles with or without serum in the present study, whereas in intact preovulatory rat follicles, hCG suppressed apoptosis after 24 h of culture (Chun *et al.*, 1994).

In the present study, in the presence of serum, dbcAMP stimulated phosphatidylserine exposure in isolated granulosa cells and in granulosa cells obtained from cultured preovulatory follicles in a dose-dependent way. Results obtained in isolated granulosa cells support the findings by others that high doses of cAMP analogues (used at saturating doses for steroidogenesis) stimulated apoptosis of granulosa cells from rat preovulatory follicles (Aharoni *et al.*, 1995) and apoptosis of human granulosa cells (Makrigiannakis *et al.*, 1999) *in vitro*. Moreover, in non-ovarian cells, cAMP stimulates apoptosis in a rat leukaemia cell line (Duprez *et al.*, 1993) and in thymocytes (McConkey *et al.*, 1992).

In contrast, the effect of dbcAMP in cultured preovulatory follicles is quite different from results obtained in cultured preantral and early antral rat follicles, as 8-bromo-cAMP did not suppress apoptosis in preantral follicles (McGee *et al.*, 1997; Hsueh *et al.*, 2000), whereas a high dose of dbcAMP inhibited apoptosis in early preantral follicles (Chun *et al.*, 1996). These contrasting results may reflect stage-dependent hormonal regulation of apoptosis during follicular development. Firstly, as underlined by McGee *et al.* (1998), the *in vivo* observation that preantral rat follicles do not ordinarily undergo apoptosis in significant numbers indicates that survival of preantral follicles may be regulated differently from survival of antral follicles. Moreover, in rats, IGF-I, interleukin 1 β (IL-1 β), LH, epidermal growth factor (EGF), fibroblast growth factor (bFGF) and growth hormone (GH) exerted a less protective effect *in vitro* on early antral follicles than on preovulatory follicles (Hsu and Hsueh, 1997). Another study performed in pig granulosa cells showed that FSH treatment protects granulosa cells isolated from small and medium-sized follicles from undergoing GnRH-mediated apoptosis, but fails to have a protective effect in granulosa cells from large follicles (Takekida *et al.*, 2000).

The results of the present study show that the lower doses of dbcAMP caused an increase in the incorporation of [3 H]thymidine into DNA without detectable anti-apoptotic effects in isolated granulosa cells. This result is consistent with the fact that FSH, which caused a modest increase in intracellular cAMP concentration, had no effect on apoptosis of isolated granulosa cells.

In our experiments, the high doses of dbcAMP caused apoptosis and, at the same time, a decrease in the incorporation of [3 H]thymidine in granulosa cells, whereas the percentage of propidium iodide-unlabelled granulosa cells

Fig. 7. Effect of increasing doses of dibutyryl cAMP (dbcAMP) on the production of (a) progesterone and (b) oestradiol in isolated rabbit granulosa cells cultured for 72 h with fetal calf serum (FCS). Data are mean $\pm$ SEM of three experiments performed in duplicate. *** $P < 0.001$ compared with control value.

(cells without a damaged membrane) did not change significantly, excluding a cytotoxic effect of dbcAMP. Moreover, the same effect was observed using another cAMP analogue, 8-bromo-cAMP (data not shown). Such an effect of dbcAMP on the incorporation of [3 H]thymidine has also been observed in human granulosa cells (Yong *et al.*, 1992) and corresponded to the decrease in the mitotic index that occurs during the atretic process (Hirshfield and Midgley, 1978).

When granulosa cell apoptosis was induced by high doses of dbcAMP in the present study, an increase in oestradiol and progesterone production was also observed. This observation was also made by Aharoni *et al.* (1995) and may be explained by the compartmentalization of steroidogenic organelles that occurs during the first 24 h of cAMP induction of apoptosis. Thus, steroidogenesis would take place in apoptotic cells if the intracellular disorganization remains moderate (Aharoni *et al.*, 1995).

The dose of 5 mmol dbcAMP l⁻¹ was supraphysiological (but not cytotoxic as shown by the percentage of propidium iodide-unlabelled cells), as it induced a ten-fold higher stimulation of progesterone secretion than did hCG. Thus, at

this dose, granulosa cells would be more vulnerable because of this hyperstimulation of steroidogenesis and could then become apoptotic. However, the dose of 2.5 mmol dbcAMP l^{-1} induced the same stimulation of progesterone production in granulosa cells as did hCG and, at the same time, induced a stimulation of apoptosis, whereas hCG did not. Thus, it can be supposed that the induction of apoptosis by 2.5 mmol dbcAMP l^{-1} was not a result of hyperstimulation of steroidogenesis.

The physiological meaning of an apoptotic effect of high concentrations of dbcAMP remains unclear. Nevertheless, if a transitory increase in cAMP were to occur in granulosa cells *in vivo*, apoptosis could be prevented by rapid desensitization of the cells, as suggested by Amsterdam *et al.* (1996).

The mechanism by which cAMP can induce apoptosis is not yet clear and different hypotheses have been suggested. In some cell lines, over-expression of cAMP-response-element-binding (CREB) protein results in apoptosis (Saeki *et al.*, 1999). Thus, an increase in the intracellular cAMP content may induce apoptosis through activation of CREB. In rat granulosa cells, cAMP-induced apoptosis involved the activation of tumour suppressor gene p53 (Keren-Tal *et al.*, 1995). cAMP appears to activate P53 via inhibition of the expression of oncoprotein Mdm2 (Hosokawa *et al.*, 1998), which inhibits P53 activity (Wu *et al.*, 1993). Makrigiannakis *et al.* (1999) suggested that cAMP-induced apoptosis in cultured human granulosa cells was mediated through downregulation of the expression of the N-cadherins expressed *in vivo* and regulated during follicular maturation.

In conclusion, in the present study it was demonstrated in the same experiment that FCS reduces the occurrence of apoptosis and also influences the effects of FSH and dbcAMP on apoptosis. It was also shown that the effect of gonadotrophins on apoptosis of preovulatory rabbit follicles is different in isolated granulosa cells and in intact follicles, showing that the regulation of apoptosis in granulosa cells appears to require paracrine interactions within the follicle. In contrast, cAMP had a biphasic effect on growth and apoptosis of granulosa cells; low doses of dbcAMP can induce DNA synthesis, whereas high concentrations induce apoptosis. The physiological meaning of this latter paradoxical effect remains to be elucidated.

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