Contribution of progesterone, follicle stimulating hormone and glucocorticoids in survival of serum-free cultured granulosa cell explants

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Abstract

To investigate the role of progesterone (P4) as a survival factor in quail granulosa cell explants, P4 content was determined under various conditions and correlated with apoptotic indexes (AIs) evaluated by 2',6'-diamidino-2-phenylindole (DAPI)-staining. Analysis of serum-free cultures from 24 to 96 h shows decreased P4 levels in the medium paralleled by increasing AI. Inhibiting apoptosis by gonadotropic support (FSH, 100 ng/ml) stimulates a 3-fold increase of the P4 level in the medium (83.49 ± 8.69 vs 26.31 ± 1.61 ng/ml in serum-free controls) together with a significant decrease in AI from $8.81 \pm 1.06\%$ in serum-free controls to $3.50 \pm 0.72\%$. Substantial evidence for P4 as an autocrine/paracrine survival factor can be inferred from experiments with aminoglutethimide (AG, 1 mM) and RU486 (20 μ M). Blocking P4 synthesis by AG causes a 2-fold increase in apoptosis from $6.08 \pm 0.67\%$ in serum-free controls to $12.53 \pm 1.60\%$. Blocking P4 receptors by RU486 causes a similar increase in AI ($3.02 \pm 0.98\%$ in serum-free controls

to $17.07 \pm 3.20\%$) and about a 50% decrease in P4. The effect of RU486 could be attenuated by exogenous P4 but not by dexamethasone indicating selective binding of P4 to the progesterone receptor. Dexamethasone treatment promotes survival without affecting P4 levels. In further support of an autocrine/paracrine action for P4 in the granulosa cells, both the A and B form of the avian P4 receptor (PR) are identified *in vivo* and *in vitro* by Western blotting. Exogenous administration of P4 only affects survival when endogenous P4 synthesis is blocked or after 48 h of serum-free culture when endogenous P4 production is very low. Because FSH also affects survival when its stimulatory effect on P4 synthesis is blocked by AG (AI decrease from $6.08 \pm 0.67\%$ in serum-free controls to $1.64 \pm 0.71\%$ in FSH+AG treated) it is proposed that (1) P4 is an autocrine/paracrine survival factor in the preovulatory granulosa and (2) FSH mediates both P4-dependent and P4-independent survival pathways.

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Introduction

Once follicles start to grow they are either selected for ovulation or, like the majority of them, they become atretic. The underlying molecular mechanism for follicle degeneration is apoptosis (or programmed cell death) as shown for all vertebrate species analysed so far (Tilly *et al.* 1997) and even for invertebrates (Criel & D'Herde 1996). Follicular atresia is initiated by programmed cell death of the somatic cells surrounding the oocyte: the granulosa cells (Tilly *et al.* 1997).

The major regulators of follicle development are gonadotropins. Gonadotropins stimulate granulosa cells to secrete various growth factors which in turn modulate ovarian steroidogenesis and other survival factors through at least three separate signalling pathways involving protein A kinases, protein C kinases and tyrosine kinases; of these

the adenylyl cyclase–cAMP–A kinase pathway is the most studied (Hsueh *et al.* 1984, Richards 1994, Chun & Hsueh 1998).

The effects of oestrogens and androgens on granulosa cell survival are well documented (Hsueh *et al.* 1984, Billig *et al.* 1994, Hsu & Hsueh 1997). Several studies suggest that P4, the major steroidal output of the preovulatory granulosa, can regulate granulosa cell proliferation (Chaffkin *et al.* 1992) and inhibit apoptosis in granulosa cultures (Luciano *et al.* 1994). The survival effect of the paracrine factor epidermal growth factor (EGF) is also mediated by P4 (Luciano *et al.* 1994) and P4 itself stimulates synthesis of the autocrine survival factor bovine fibroblast growth factor (bFGF) in rat granulosa cells (Peluso & Pappalardo 1999). For the corpus luteum, another target tissue of P4 action, it was recently shown that the survival effect of P4 involves downregulation of

Fas expression, a member of the cell death receptor family (Kuranaga *et al.* 2000).

In support of a direct ovarian action for P4, specific P4 receptors (PRs) existing as A and B forms have been demonstrated in the avian ovary with the highest amount in the F1 follicle (Yoshimura & Bahr 1991, Isola *et al.* 1987a). Isola and colleagues (Isola 1987, Isola *et al.* 1987a,b) describe a nuclear localization of the PR in chick oviduct and some unoccupied PRs in the cytosol (Isola *et al.* 1986). As conflicting data exist about the localization of unoccupied receptors, Clark & Markaverich (1988) suggested that the PRs in the nucleus are in a state of equilibrium with those in the cytoplasm, which results in a small percentage of receptors being located in the cytoplasm. Occupied PRs are always found in the nucleus where they fulfil their role as a transcription factor. The central role of P4 and the PR in mammary development is underlined by the many pleiotropic reproductive abnormalities such as anovulation, limited mammary gland development, hyperplasia, as well as neuroendocrine defects, found in PR knockout mice (Lydon *et al.* 1995, Chappell *et al.* 1997). In rat follicles, PR mRNA is not expressed until after the pre-ovulatory luteinising hormone (LH) surge (Park & Mayo 1991, Natraj & Richards 1993). Despite this absence of the PR prior to the LH surge, P4 is able to maintain the viability of rat granulosa cells possibly through a membrane P4-binding protein (Peluso 1997, Peluso & Pappalardo 1999).

In the present study an *in vitro* model was used consisting of serum-free culture of granulosa cell explants derived from the vegetative pole of the largest pre-ovulatory follicle. In this system the granulosa epithelial explants remain sandwiched between their native basement and vitelline membrane, in this way, cell–cell interaction like metabolic exchanges and transport of small molecules through gap junctions (D'Herde & Leybaert 1997, Farioli-Vecchioli *et al.* 2000) is maintained. This model thus closely resembles normal *in vivo*-like tissue structure.

In the present study it was analysed whether (1) P4 acts as an important component of the follicle stimulating hormone (FSH)-stimulated anti-apoptotic cascade, (2) the survival effect of FSH is entirely P4-dependent. Therefore after detecting PR isoforms at various culture stages, the correlation was examined between apoptotic indexes and P4 levels under basal serum-free conditions, under gonadotropic support and in conditions where P4 synthesis was inhibited or its effect was abrogated through blockade of its receptors.

In addition, the role of dexamethasone in survival was addressed. Although it is known that glucocorticoids highly augment cAMP-stimulated progesterone production in several granulosa models (Hosokawa *et al.* 1998), its regulatory role in survival is not well documented (Makrigiannakis *et al.* 2000).

Materials and Methods

Animals

Granulosa cell explants were prepared from the largest (F1) pre-ovulatory follicle of the adult Japanese quail (*Coturnix coturnix japonica*). The animals were reared under continuous artificial illumination, with food (fresh lettuce and complete breeding food, Biofor AVEVE, Belgium) and water *ad libitum*. Animal care procedures were conducted in accordance with the guidelines set by the European Community Council Directives 86/6091 EEC.

Isolation and culture of granulosa cell explants

The monolayered granulosa layer from the largest pre-ovulatory follicle (F1) was isolated from the follicle wall in Krebs–Ringer solution according to the technique described by Gilbert *et al.* (1977). Farioli-Vecchioli *et al.* (2000) defined three regions in quail granulosa explants which are analogous to those defined in chicken by Tischkau & Bahr (1996). The area of the transition region between the animal and vegetal pole which is in close contact with the animal pole contains several cell proliferative factors which inhibit P4 production by chicken granulosa cells (Tischkau & Bahr 1996). Therefore the animal pole and transition region were selectively removed from the rest of the granulosa cell (GC) explant. The morphology, mitotic rate and other factors in these latter regions of the granulosa layer differ from those in the vegetal pole region and probably represent a subpopulation as described for hen (Marrone & Crissman 1988) and rat granulosa cells (Peluso 1997). The remaining granulosa from the vegetal pole region of *circa* 5.8 cm² was divided into smaller squares of *circa* 4 mm², followed by culture in 35 mm Petri dishes under serum-free conditions in a 38 °C incubator. Explants were cultured for various time periods depending on the experiment type. Culture times of 24 h were used for evaluation of AG, RU486, dexamethasone and FSH effects; to correlate apoptotic indexes with P4 levels in the medium granulosa explants cultured for 24, 48, 72 and 96 h were analysed. GC explants were cultured for up to 72 h for P4 rescuing experiments and PR Western blotting. Explants were cultured in filter-sterilized M199 (Sigma, cat. no. M-0393, Bornem, Belgium) at pH 7.4, supplemented with 0.1% w/v bovine serum albumin fraction V (Sigma, cat. no. A-4503, Bornem, Belgium), 10 mM HEPES (Acros, Geel, Belgium), 1% v/v penicillin–streptomycin (Paisley, UK) and 4 mM sodium hydrogen carbonate.

Reagents and hormones

Sheep pituitary FSH (100 ng/ml, Sigma, Bornem, Belgium), was dissolved in 0.9% NaCl and aminoglutethimide (Sigma, Bornem, Belgium) was dissolved in

sterile DMSO (Sigma, Bornem, Belgium). Progesterone (Sigma, Bornem, Belgium) and RU486 (Mifepristone or Mifegyne, a generous gift from Laboratoires Exelgyn, Paris, France) were dissolved in MeOH and EtOH respectively. Dexamethasone (Sigma, Bornem, Belgium) was dissolved in sterile DMSO. Dosages were chosen based on dose–response experiments; the lowest dose with effect was used. In control cultures (M199) the medium was rinsed and refreshed with M199 as in the treated counterparts. There was no addition of vehicles to controls as the concentration of all vehicles was always kept below 0.1% in the corresponding treated fraction. It was found in the present quail granulosa cell explants that below this concentration the used vehicles had no influence on P4 levels and cell death (data not shown).

Staining and quantification of apoptotic nuclei

GC explants were rinsed in phosphate-buffered saline (PBS, 10 mM, pH 7.4), fixed for 20 min in 4% formaldehyde in PBS, again rinsed in PBS and stained using a 1/1000 solution of 2',6'-diamidino-2-phenylindole (DAPI) in PBS. Mounted GC explants were examined under a Leica DM IRB/E inverted microscope ($\times 63$ objective, 1.0 zoom) and apoptotic cells were identified by their characteristic fragmented chromatin masses. Small groups of apoptotic bodies were counted as remnants of one apoptotic cell. Apoptosis was expressed as the number of apoptotic nuclei per number of total nuclei counted in the same microscopic field. This apoptotic index (AI) was averaged for 10 fields giving a total number of about 1500 cells counted per treatment.

Progesterone (P4) assay

From *in vitro* incubation studies, it has been clarified that the primary source of P4 in avian species is the granulosa cell of the largest (F1) follicle, the one destined to ovulate next. In the smaller pre-ovulatory follicles, the second (F2) and the third (F3) largest follicles, P4 is rapidly metabolised due to their significant higher amounts of conversion enzymes in the theca cells (Huang & Nalbandov 1979, Mori *et al.* 1984, 1985, Mori 1987).

Progesterone was measured in spent medium using an automated chemiluminescence system (Chiron Diagnostics ACS:180 P4 assay), a competitive immunoassay with a minimum detectable concentration of 0.11 ng/ml (0.35 nM). Samples were run in several assays. The intra-assay variation is given by a covariance of 3.3% for an average value of 5.3 ng/ml and 7.9% for 10.9 ng/ml; the interassay variation is 10.1% for 6.3 ng/ml and 10.4% for 14.0 ng/ml. The actual precision, determined by both intra- and interassay variation, is given by a covariance of 5.4% for 8.1 ng/ml and 4.9% for 19.6 ng/ml.

Figure 1 DAPI staining of an F1 granulosa cell explant consisting of a monolayered granulosa cell explant sandwiched between the basement and vitelline membrane. Arrows show condensed nuclear chromatin masses characteristic of apoptotic bodies. Scale bar, 10 μ m.

Identification of the progesterone receptor by Western blot analysis

Cell explants were lysed in 200 μ l lysis buffer (10 mM Tris–HCl pH 7, 1% NP40, 200 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF)). Cleared cell extracts (30 μ g cellular protein) were loaded on a 15% SDS–polyacrylamide gel. After electrophoretic separation and blotting to a nitrocellulose membrane, the progesterone receptor (PR) was detected using the aPR22 monoclonal antibody (Affinity Bioreagents, Inc.), which reacts with both the 80 kDa A form and the 110 kDa B form of avian PR, in a 1/1000 dilution in blocking buffer (5% dry milk in PBS containing 0.3% Tween 20) and developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, UK).

Statistical analysis

Statistical analysis was done using the statistical program SPSS 6.1. (SPSS Inc.). Statistical significance was tested with the Wilcoxon rank sum test, a non-parametric test for paired samples. *P* values less than 0.05 were considered significant. Values represent means $\pm$ S.E.M. of at least four independent experiments.

Results

The effect of medium change and FSH on apoptosis

Apoptotic indexes of DAPI-stained granulosa explants were determined after 24, 48, 72 and 96 h in culture (Fig. 1), P4 levels were defined in the corresponding spent medium. Figure 2 summarises the data obtained, namely, a significant increase in AI with increasing culture time up

Figure 2 Effect of medium change on apoptotic indexes and P4 concentrations at the 24, 48, 72 and 96 h culture stage of serum-free culture. Data represent means $\pm$ S.E.M. Significant differences ($P < 0.05$) are observed between changed and unchanged media conditions and between culture stages (except for 96 h: $n = 1$). (◆), AI – medium not replaced; (▲), P4 (ng/ml) – medium not replaced; (■), AI – medium replaced every 24 h; (●), P4 (ng/ml) – medium replaced every 24 h.

to 72 h followed by a decrease at the 96 h culture stage ($P < 0.05$). This is associated with a continuous decrease of P4 concentrations in the medium ($P < 0.05$). When the medium was changed every 24 h, thus washing out the P4, the AIs are significantly higher and the P4 concentrations significantly lower ($P < 0.05$). The AI peak observed at 72 h when the medium remained unchanged was not observed when the medium was changed every 24 h.

As the medium is replaced every 24 h, the P4 values at the different time stages are the actual amounts (secretion minus consumption) present between the given culture

stages. From the P4 levels it can be inferred that P4 secretion continues in serum-free culture: 1.12 ± 0.12 ng/ml between 24 and 48 h; 0.37 ± 0.18 ng/ml between 48 and 72 h. After 72 h the P4 level becomes negligible (< 0.3 ng/ml).

To further assess the role of P4 in apoptosis, P4 concentrations and apoptotic indexes were determined in cultures wherein steroidogenesis was stimulated by FSH. When the medium was supplemented with FSH (100 ng/ml), P4 levels were increased 3-fold from 26.31 ± 1.61 ng/ml in serum-free controls to 83.49 ± 8.69 ng/ml ($P < 0.05$, Fig. 3) together with a

Figure 3 The dose-dependent effect of FSH on the percentage apoptosis after 24 h serum-free culture. Hatched columns represent AI, open columns represent P4 levels. Data represent means $\pm$ S.E.M.

Figure 4 The apoptosis-inducing effect of AG (1 mM) after 24 h serum-free culture. Hatched columns represent AI, open columns represent P4 levels. Data represent means $\pm$ S.E.M.

significant decrease in apoptotic indexes from $8.81 \pm 1.06\%$ in serum-free controls to $3.50 \pm 0.72\%$. The effect of FSH was dose dependent (Fig. 3); 500 ng/ml FSH reduced the AI significantly to $1.37 \pm 0.61\%$, 1000 ng/ml made no further contribution to survival despite the further increase in P4 levels in the medium. But as the AIs were close to 0%, it was difficult to measure the effect; in a single case, where AI equalled 3.07% with 500 ng/ml, 1000 ng/ml FSH decreased the AI another 2- to 3-fold. To examine whether P4 is a direct cause of cell survival or is simply associated with cell survival, the effect of several inhibitors in the P4 mode of action was examined.

RU486 (Mifepristone or Mifegyne) and aminogluthetimide (AG)

When the granulosa cell explants were incubated for 24 h with the P4 synthesis inhibitor aminogluthetimide (AG, 1 mM), the apoptotic process was enhanced 2-fold from $6.08 \pm 0.67\%$ to 12.55 ± 1.60 , together with a significant decrease in P4 levels in the medium ($P < 0.05$, Fig. 4). RU486 (20 μ M) was also effective in increasing the apoptotic indexes by blocking the P4 receptors: from $3.02 \pm 0.98\%$ in serum-free controls to $17.07 \pm 3.20\%$ ($P < 0.05$; Fig. 5). This was accompanied by a 50% decrease in P4 concentrations from $35.83 \pm 3.23\%$ in serum-free controls to $16.88 \pm 3.59\%$ in RU486-treated explants. To rule out the possibility that RU486 acts through binding to glucocorticoid receptors, cultures were additionally supplemented with either dexamethasone or P4. Dexamethasone (Dex) was not able to reverse the effect of RU486 whereas P4 (320–640 nM) in combination with RU486 significantly decreased AI to control values in a

dose-dependent manner. Dexamethasone (0.1, 1 and 10 μ M) on its own decreased AI significantly: for 1 μ M: from $3.02 \pm 0.98\%$ in serum-free controls to $0.99 \pm 0.40\%$ in Dex-treated explants (Fig. 5, $P < 0.05$). The values for the other concentrations of Dex are similar to this result. A concentration of 0.01 μ M did not affect apoptosis. P4 levels were unchanged compared with serum-free controls.

Exogenous progesterone (Pex) and the involvement of endogenous P4

As expected from the above results, application of Pex should result in a direct effect on viability. Different concentrations (640, 3200 and 6400 nM) were used during 24 h incubations and no significant inhibition of apoptosis compared with serum-free controls occurred although the P4 concentration in the medium was significantly increased from 26.31 ± 1.61 ng/ml in serum-free controls to 177.00 ± 15.56 (Fig. 6); 832.93 ± 72.40 and 1791.00 ± 73.76 ng/ml ($P < 0.05$; data not shown) respectively.

Inhibiting the endogenous P4 production for 24 h by simultaneously applying 1 mM AG and Pex (640 nM) resulted in a significant 2-fold decrease in AI compared with AG experiments (12.53 ± 1.60 vs $7.28 \pm 2.04\%$). Compared with serum-free controls, the AG+Pex experiments did not enhance viability despite the significant, almost 10-fold, increase in P4 concentration in the medium (Fig. 6).

Adding P4 (640–3200 nM) between 48 and 72 h, when endogenous P4 production is low (6.19 ± 0.66 vs 26.31 ± 1.61 ng/ml at 24 h) results in a significant decrease in AI compared with serum-free controls ($P < 0.05$,

Figure 5 The effects of RU486 (20 µM) and Dex on apoptosis after 24 h serum-free culture. Hatched columns represent AI, open columns represent P4 levels. Data represent means ± S.E.M.

Fig. 7). Assaying Pex at different culture stages (24, 48, 72 and 96 h) in M199 medium without granulosa explants shows that there is no spontaneous degradation of P4 (data not shown).

FSH combined with aminogluthetimide: a possible P4-independent survival pathway?

As FSH is able to block apoptosis and to stimulate P4 secretion and Pex only affects survival when endogenous secretion is experimentally inhibited or falls spontaneously in prolonged culture, then the existence of a FSH-induced

survival pathway acting independently of P4 can be hypothesised. Experiments were designed to test the hypothesis: 24 h cultures supplemented with FSH (100 ng/ml) in combination with AG (1 mM). Surprisingly, results show a significant decrease in AI compared with serum-free controls (from $6.08 \pm 0.67\%$ in serum-free controls to $1.64 \pm 0.71\%$) even though there is a significant decrease in P4 from 26.31 ± 1.61 to 8.73 ± 1.99 ng/ml. The decrease in AI is comparable to the decrease obtained by FSH supplementation alone although in the latter the P4 production is about eight times higher (Fig. 8).

Figure 6 The effects of Pex (640 nM) and AG (1 mM) after 24 h serum-free culture. Hatched columns represent AI, open columns represent P4 levels. Data represent means ± S.E.M.

Figure 7 The effect of 24 h P4 supplementation (320 nM, 640 nM, 3200 nM) on apoptosis after 48 h of serum-free culture. Hatched columns represent AI. Data represent means $\pm$ S.E.M.

Identification of the progesterone receptor by Western blotting

The presence of the A (PR-A) and B (PR-B) form of the PR was investigated at 0, 24, 48 and 72 h of serum-free culture by Western blotting using a monoclonal antibody against both forms of the avian PR. Figure 9 shows a strong signal at all culture stages tested of 110 kDa representing PR-B and a weak signal representing the 78 kDa PR-A.

Discussion

The present study examined whether P4 acts as an autocrine/paracrine survival factor in serum-free cultured

granulosa explants isolated from the largest pre-ovulatory follicle of the quail. The use of an avian model for endocrine studies is interesting because of the hierarchical arrangement of the pre-ovulatory follicles, which allows an easy identification of the maturational stage of the follicle and prediction of time of ovulation. Secondly, the theca (the site of androgen and oestrogen production) and granulosa (the source of P4) layers can easily be separated without contamination (Bahr 1990). Apoptosis was assessed by DAPI staining disclosing the typical morphological features of apoptotic nuclei. Apoptotic morphological features were also confirmed at the ultrastructural level in 72 h cultured quail granulosa explants (D'Herde *et al.* 2000). It was previously shown for avian granulosa that methods detecting chromatin condensation and

Figure 8 The effect of FSH (100 ng/ml) combined with AG (1 mM) after 24 h serum-free culture. Hatched columns represent AI, open columns represent P4 levels. Data represent means $\pm$ S.E.M.

Figure 9 Western blot of the avian PR in granulosa cell explant of the largest preovulatory follicle (F1) at 0, 24, 48 and 72 h.

fragmentation are more reliable for quantification of apoptosis than methods using *in situ* end labelling (D'Herde *et al.* 1994). Granulosa cell explants cultured up to 96 h, show an increase of apoptotic indexes peaking at 72 h whereafter a subpopulation was selected which is resistant to gonadotropin withdrawal (D'Herde & Leybaert 1997).

In the present study P4 levels were compared with apoptotic indexes. An inverse relationship was found between P4 levels and AI up to 72 h. At 96 h, however, when AI declines in the unchanged cultures, P4 levels are not restored probably because of dedifferentiation or reduced steroidogenic capacities of the surviving cells. One has to bear in mind that the P4 found in the medium results from secretion and uptake by the cells for further metabolism as there is no spontaneous degradation of P4 in the medium. By replacing the medium every 24 h and thus washing out P4, one obtains some idea of the actual production during the different culture stages, assuming that the consumption of P4 is constant. The data indicate that quail granulosa cells remain steroidogenically productive until 72 h of serum-free culture, after that the P4 level becomes negligible. Peddie *et al.* (1994) cultured chicken granulosa cells for up to 72 h without changing the medium daily and found that basal P4 accumulation in the medium increased over 24–48 h in culture and then tended to decline. The P4 secretion *in vitro* was not expected since *in vivo* the collected F1 granulosa cells (2 h before ovulation) will be poised to deteriorate following follicle rupture at ovulation.

To determine whether P4 can act as a survival factor in the granulosa system it was first investigated whether the progesterone receptor (PR) is expressed in the F1 granulosa explants. Western blot analysis was performed using a monoclonal antibody against both the A and B forms of chicken PR. Yoshimura & Bahr (1991) described the presence of PR in granulosa cells of F1 of the domestic hen. This phenomenon is paralleled by the higher P4 production through the follicle hierarchy (see Materials and Methods) indicating a possible important target function of the pre-ovulatory granulosa layer for

P4 in addition to the more traditionally accepted target tissues of P4 like the uterus and the mammary gland. The 110 kDa PR-B and 78 kDa PR-A isoforms were present in the granulosa explants from the F1 follicle at all culture stages tested (0, 24, 48 and 72 h). PR-A is known to appear sometimes as a very faint band (D Toft, personal communication). The presence of both PR isoforms is in accordance with the findings for granulosa cells of the domestic hen (Yoshimura & Bahr 1991). In most mammalian models PR-A is transcriptionally inactive and acts as a potent transdominant repressor of PR-B-mediated transcription and of other steroid receptor activity (Vegeto *et al.* 1993). However, although both A and B forms of mammalian and avian PR are very similar, Giangrande *et al.* (1997) showed that the mechanism of action of the PR in both models are quite different due to the lack of the repressor function of PR-A in chicken. The Western blot analysis also reveals a large amount of background probably consisting of heat-shock (hsp70, hsp90) and other proteins including p50 and p53, known to be associated with the inactivated chicken PR (Schowalter *et al.* 1991). In Fig. 9, the band above 110 kDa in lanes 1 and 2 most likely represents PR still bound to hsp90 or other proteins.

The higher apoptotic indexes obtained by washing out P4 are in agreement with the hypothesis of P4 being a survival factor in the granulosa explants. As described by Hsueh *et al.* (1984), FSH regulates progesterone biosynthesis by modulating the activities of various steroidogenic enzymes. Accordingly, when the medium was supplied with FSH, an increase in P4 levels in the medium and an inhibition of apoptosis when compared with serum-free controls was observed. Both effects of FSH on steroidogenesis and survival are dose dependent. Although pre-ovulatory follicles are largely under the control of LH via the dominant expression of the LH receptor, FSH was used to stimulate P4 production as FSH was utilised successfully in earlier studies to inhibit apoptosis (D'Herde & Leybaert 1997, D'Herde *et al.* 2000). In these papers, several effects of FSH with respect to apoptosis signal transduction are well documented. As FSH affects a myriad of processes in cultured granulosa cells (reviewed by Hsueh *et al.* 1984), which could also be responsible for apoptosis inhibition, dependent or not on P4, it was necessary to further elucidate the involvement of P4 in FSH-mediated cell survival. Additional evidence for the apoptosis-inhibiting role of P4 came from the experiments with AG and RU486. When P4 synthesis was blocked by inhibiting the side-chain cleavage step from cholesterol to pregnenolone by AG or when the P4 receptors are occupied by RU486, the AI are higher than in serum-free controls. This means that apoptosis is induced by omitting protection of P4 and moreover, that the hormone operates through its receptors. Blocking of the receptors by RU486 was accompanied by a decrease in P4 levels measured in the medium. This direct action of RU486 on luteal

function, besides their antagonism at the progesterone receptor level was previously observed by Singh *et al.* (1988) in adult female rats. Analogous apoptosis induction was reported previously in isolated rat granulosa cell cultures (Luciano *et al.* 1994, Peluso and Pappalardo 1999) as well as in human granulosa cells (Makriganakis *et al.* 2000). Although RU486 is a P4 receptor antagonist, a partial agonist function is described depending on the cell type used (Vegeto *et al.* 1996, 1999, Nordeen *et al.* 1995, Omigbodun *et al.* 1997). Paradoxical agonist effects of the PR antagonist RU486, a persistent clinical problem, were not observed at the 24 h culture stage. As P4 and RU486 are known to have antiglucocorticoid action as well, RU486-treated cultures were additionally supplemented with dexamethasone (Dex), a potent glucocorticoid hormone analogue, or P4 to exclude influence of the glucocorticoid receptor (GR). It can be concluded that, if the glucocorticoid receptor agonist Dex was not able to reverse the effect of RU486 while P4 could, P4-mediated survival occurs via the progesterone receptor. Supposing that both P4 and RU486 operate through the glucocorticoid receptor then both treatments should result in an increase of AI, which is not the case for P4. Dex suppressed apoptosis significantly without affecting P4 levels in contrast to its apoptosis-inducing effect in thymocytes, pointing to a separate survival pathway in the granulosa model. This apoptosis-inhibiting effect of Dex is found in several other models, some in conjunction with a P4-survival pathway, such as in mouse mammary glands (Feng *et al.* 1995), neutrophils (human: Liles *et al.* 1995; rat: Meagher *et al.* 1996, Nittoh *et al.* 1998), mouse uterine epithelial cell (Jo *et al.* 1993) and in rat hepatoma cells (Yamamoto *et al.* 1998, Evans-storms & Cidlowski 2000). In serum-free cultured human granulosa cells Dex treatment did not affect survival (Makriganakis *et al.* 2000). Hsueh *et al.* (1984) describes the ability of Dex to enhance P4 biosynthesis in cultured rat granulosa cells but this was tested in combination with FSH supplementation. However, in the present study, no difference was observed in P4 levels in the medium of serum-free controls and Dex-treated explants.

As blocking of the P4 receptors causes the cells to go into apoptosis and FSH-stimulated P4 production causes survival, one would expect that exogenous supplied P4 (Pex) would invariably promote cell survival. This is the case for rat large granulosa cells where administration of Pex is sufficient to prevent apoptosis *in vitro* and where this effect could be attenuated by RU486 but not by AG (Luciano *et al.* 1994). Although AG and RU486 induce apoptosis in quail granulosa explants, no significant protection on viability by Pex alone can be observed in the 24 h cultures as compared with serum-free controls. Analogous results were found by Rueda *et al.* (2000) for bovine luteal cells. Both AG and RU486 treatment induced apoptosis, whereby the AG effect could be attenuated by Pex. Pex treatment alone, however, did not affect survival

as compared with serum-free controls. In accordance with the present study basal P4 levels under serum-free conditions were high (>100 ng/ml); this in contrast to the low P4 levels (6.30 ± 0.70 ng/ml) in rat granulosa cells where Pex has a survival effect (Luciano *et al.* 1994). As an explanation as to why supplementation with Pex does not affect survival one can argue that P4 receptors are saturated in the 24 h serum-free cultured granulosa explants in line with the P4 output at this stage (26.31 ± 1.61 ng/ml; for reference (Onagbesan & Peddie 1988): in quail serum P4 levels around 2.52 ng/ml are the threshold value for inducing ovulation), so that exogenous P4 has no effect as long as there is no substantial drop in endogenous P4 secretion. Further evidence for this state of saturation at the 24 h culture stage is shown in the experiment where P4, added at 48 h when endogenous P4 levels are substantially decreased (6.19 ± 0.66 versus 26.31 ± 1.61 ng/ml at 24 h), indeed influenced survival. Secondly, Pex can have a suppressive effect on the expression of its own receptors (Clark & Markaverich 1988). To test these two concepts experiments were designed with combinations of AG and Pex. Where Pex had no effect on survival, Pex in combination with AG did have an effect on survival. The fact that Pex could reverse the AG inhibition of P4 synthesis confirms the specificity of AG and the anti-apoptotic capacity of P4 in non-saturated PR conditions. Moreover it proves that in the present study AG did not exert a general toxic effect.

As FSH is able to stimulate both P4 and survival, it could be that (1) FSH triggers expression of P4 receptors as in rat granulosa cells (Natraj & Richards 1993), probably under cAMP control, or (2), the FSH effect is not entirely P4 dependent. FSH-stimulated production of oestrogens, a known survival factor in other ovarian model systems (Peluso *et al.* 1981, Hsueh *et al.* 1984, Billig *et al.* 1994, Vegeto *et al.* 1999), can be excluded as this steroid is not produced in the avian granulosa cells but in the theca cells (Bahr 1990). Without excluding a mediating role for the P4 receptors, evidence for a P4-independent survival pathway came from experiments where AG is combined with FSH. The stimulating effect of FSH on P4 secretion was inhibited by AG but its protecting effect was still present meaning that FSH can inhibit apoptosis even when P4 synthesis is blocked. In rat granulosa cells the only P4-independent survival pathway elucidated hitherto is the effect of cell contact through adhesion type junctions (Peluso *et al.* 1996).

In conclusion the data presented in this article are in line with the hypothesis that at least two distinct survival pathways maintain granulosa cell viability in the studied *in vivo*-like model system. First a P4-dependent survival pathway under gonadotropic control, whereby P4 acts as an autocrine/paracrine survival factor mediating its effect at receptor level. Secondly a P4-independent survival pathway influenced either by FSH or by glucocorticoids.

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