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Decreased Intracellular Potassium Levels Underlie Increased Progesterone Synthesis During Ovarian Follicular Atresia[1]

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ABSTRACT

More than 99% of ovarian follicles are lost by a degenerative process known as atresia, a phenomenon characterized by apoptosis of granulosa cells. Uniquely, dying granulosa cells also greatly increase their progesterone biosynthesis while reducing estrogen production. Recent studies have documented a dramatic decrease in intracellular K^+ concentration during apoptosis that plays an important role in regulating apoptotic enzymes. However, it is unclear whether this ionic change affects related processes such as the change in steroidogenesis in dying granulosa cells. To explore this question, granulosa cells were cultured in hypotonic medium, which initially swells the cells. The cells respond by extruding K^+, which we have documented by fluorescence spectrophotometry. The K^+ efflux osmotically draws water out the cell, returning it to a near normal volume (as measured by flow cytometry). The result is a cell of normal size with a decreased intracellular K^+ concentration. FSH stimulation of these cells caused an increase in progesterone biosynthesis. This response was enhanced at higher doses of FSH, although basal progesterone production was not affected, suggesting that K^+ levels may affect the gonadotropin-signaling pathway. No increase in steroidogenic acute regulatory or cholesterol side-chain cleavage cytochrome P450 mRNA was detected, although cAMP production was enhanced. These results suggest that the loss of intracellular K^+ by apoptotic granulosa cells greatly facilitates FSH-stimulated progesterone production.

apoptosis, estradiol, follicle, follicular development, granulosa cells, progesterone

INTRODUCTION

More than 99% of ovarian follicles are lost by a degenerative process known as atresia. Early work on atresia identified several structural alterations associated with this process, such as the proximal degeneration of the granulosa cell layer [1] and the formation of pyknotic nuclei [2, 3]. In addition, functional changes were detected, including an increase in progesterone production with a concomitant decrease in estrogen synthesis [4–7]. More recent studies on atresia have shown that the individual follicle cells, in particular the granulosa cells, die by apoptosis [6, 8–14]. Granulosa cell apoptosis has emerged as a classic model of cell death, engaging all the major apoptotic systems such as the caspase cascades, endonucleases, and the bcl-2 family of apoptotic modulators. However, granulosa cells are unique because as they begin to die they produce large amounts of progesterone while shutting down estrogen synthesis [4-7].

The increased progesterone production by dying granulosa cells has been demonstrated in both primary cells [6] and immortalized cells [15]. The importance of this process may be underscored by the considerable efforts of the cell to insure continued steroidogenesis as they die. For example, most of the steroidogenic machinery responsible for progesterone production is located within mitochondria. In granulosa cells undergoing apoptosis, the mitochondria are sequestered to the perinuclear regions [16], away from the apoptotic blebs [17]. Although the mitochondria play an important role early in apoptosis in many examples of cell death, there is no evidence of sequestration in these models. Given the extent of rearrangement this entails, the cell apparently expends a considerable amount of energy to exclude the steroidogenic machinery from the blebbing portion of the cell, which strongly indicates that the production of progesterone is a critical function of the granulosa cells en route to death.

Recent developments in the understanding of the apoptotic process in thymocytes and other types of cells have indicated that there is considerable K^+ loss from the intracellular compartment as a cell begins to die [18-27]. Mechanistic studies in both thymocytes and granulosa cells have indicated that the reduction in intracellular K^+ concentration, $[K^+]_i$, is critical for the activation of key components of the death machinery, such as the caspase cascade and apoptotic nucleases [18, 19, 27]. In the present study, we explored the possibility that these changes in $[K^+]_i$ are responsible for the functional changes in steroidogenesis seen in dying granulosa cells.

MATERIALS AND METHODS

Animals

Immature female Sprague-Dawley rats were raised in-house and provided with food and water ad libitum. At 21-23 days of age, rats were injected s.c. with 10 IU eCG (provided by Dr. A.F. Parlow and the National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program). Animals were killed 48 h later by cervical dislocation. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of North Carolina and were performed in accordance with the guidelines set forth in the *NIH Guide for the Care and Use of Laboratory Animals*, published by the Public Health Service.

Cell Culture and the Reduction of Intracellular K^+ Levels

For a given experiment, rats were killed, the ovaries were removed and cleaned of surrounding fascia, and granulosa cells were harvested by needle puncture [6, 28]. All granulosa cells from a given set of animals were pooled and disbursed into the cultures prepared for that day. In

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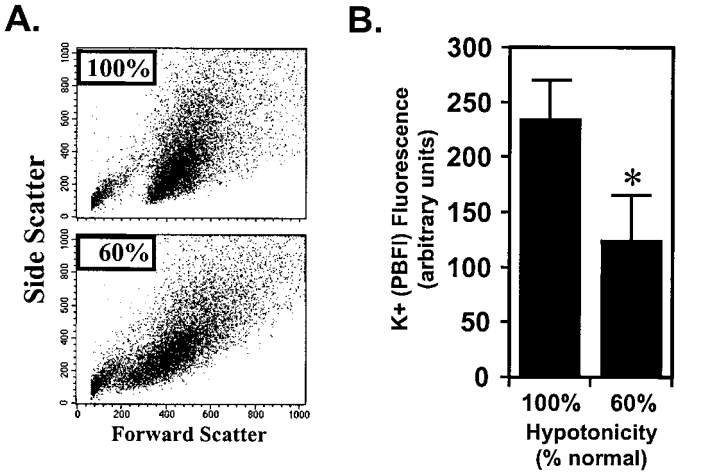

FIG. 1. Cell size and K^+ levels in granulosa cells in 100% and 60% medium. Granulosa cells from Day 2 eCG-primed rats were cultured for 4 h in serum-free McCoy 5a medium (100%) or medium diluted with H_2O to 60% of normal. The 60% medium was used as a maximal dilution because substantial necrosis was seen with greater dilutions. **A**) Dot plots, obtained by flow cytometry, indicating relative cell size (forward scatter). Following a 4-h incubation in 100% or 60% medium, cells were fixed in 70% ethanol, washed in PBS, stained with PI, and analyzed. **B**) PBFI fluorescence (intracellular K^+ levels). One hour before the end of culture, PBFI-AM was added to a final concentration of 5 μM. Fluorescence of the cells was quantitated using a fluorescence spectrophotometer. Results are the mean ± SEM of five separate experiments (*$P < 0.05$).

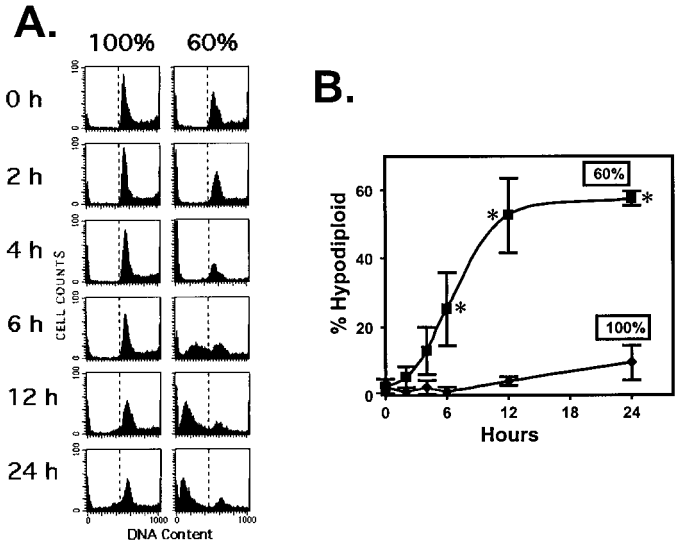

FIG. 2. Single-cell analysis of the kinetics of apoptosis (DNA degradation) in granulosa cells cultured in 100% and 60% medium. Granulosa cells were cultured in serum-free medium (100%) or medium diluted with H_2O to 60% of normal for the indicated time prior to analysis of DNA content by flow cytometry. **A**) A representative DNA histogram depicting the DNA content (PI fluorescence) of cells. The dotted line indicates the division between diploid cells (right side of the line) and hypodiploid cells (left side). **B**) Quantitation of the DNA histograms. The results are the mean ± SEM from three separate experiments (*$P < 0.05$ compared with cells cultured the same amount of time in 100% medium).

each repeat experiment, a separate set of animals were killed on a different day. Intracellular K^+ levels were experimentally reduced by dilution of the culture medium with H_2O, as previously shown with lymphocytes [29]. Cells were cultured (0.5×10^6 cells in 0.5 ml of serum-free McCoy 5a medium diluted as described in the individual experiments) in 12- × 75-mm polystyrene culture tubes and incubated at 37°C (95% air, 5% CO_2) for the indicated times. All cultures contained 0.1 μM 4-androsten-17β-ol-3-one-17-propionate (testosterone propionate) to serve as substrate for estrogen biosynthesis. Samples for analysis of steroid production were prepared in duplicate, and the mean was taken as the measurement for that experiment. All other cultures were single measurements for a given experiment.

To insure the effectiveness of this model for reducing intracellular K^+, levels of this ion in granulosa cells were assessed using the K^+-sensitive dye PBFI-AM (the cell-permeant acetoxylmethyl ester of potassium-binding benzofuran isophthalate; Molecular Probes, Eugene, OR). PBFI-AM was added to a final concentration of 5 μM 1 h prior to the end of culture [18, 19, 30]. Following incubation, the cells were washed and resuspended in isotonic PBS (i.e., diluted to an equivalent amount as the respective culture medium). The fluorescence of each population was then quantified by fluorescence spectrophotometry (excitation = 340 nm, emission = 505 nm), and the results are presented as arbitrary fluorescence units.

Flow Cytometry

Granulosa cells were cultured at 1×10^6 cells/ml in serum-free McCoy 5a medium for the indicated times. Cells were vortexed vigorously to detach them before fixing in 70% ethanol and storage at 4°C. For analysis, cells were washed in ice-cold PBS and resuspended in PBS containing 20 μg/ml propidium iodide (PI), 1 mg/ml RNase A. Following a 10-min incubation at room temperature, analysis for cell size and/or DNA content was performed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Immunoassays

Following culture, medium was removed and stored at −20°C until analyzed by radioimmunoassay for steroid content. Steroid RIAs were performed as previously described using ^{3}H-steroid and antibody (AB317 and AB333; Chemicon, Temecula, CA) [6, 31]. For analysis of cAMP production, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) was included in cultures to inhibit phosphodiesterases. Following culture, cells were lysed in 0.1 N HCl and assayed immediately or stored at −20°C until analysis. Cyclic AMP was measured using an enzyme immunoassay (Assay Designs, Ann Arbor, MI) according to the instructions of the manufacturer.

Reverse Transcription Polymerase Chain Reaction

Levels of mRNA encoding for steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage cytochrome P450 ($P450_{scc}$) were analyzed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR). Granulosa cell RNA was isolated using Trizol (Sigma, St. Louis, MO), precipitated, washed in 100% ethanol, and resuspended in H_2O. The RNA was reverse transcribed into cDNA using random oligonucleotide hexamers and Superscript II reverse transcriptase (Gibco BRL, Life Technologies, Rockville, MD). The cDNA was precipitated, washed in ethanol, and resuspended in water. The cDNA was then subjected to PCR using primers specific for StAR (forward: CCTCCCAACTCCCTACCACTT, reverse: TCTGCCATACCTTTTTCCTTGG), $P450_{scc}$ (forward: TCACAAGCCAGCATAAGGAG, reverse: GCAGCCT-

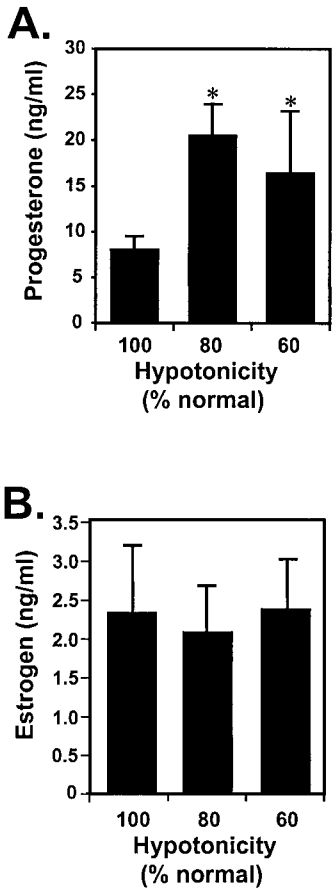

FIG. 3. Steroidogenesis in FSH-stimulated granulosa cells cultured in media of increasing dilution. Granulosa cells were cultured in the presence of 50 ng/ml FSH for 4 h in medium diluted to 100%, 80%, or 60% of normal. Medium was removed, and steroids were quantitated by RIA. **A**) Progesterone levels. **B**) Estrogen levels. Data represent the mean ± SEM from three separate experiments (*$P < 0.05$ compared with cells cultured in 100% medium).

GCAATTCATACAG), and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (forward: GGAGCCAAACGGGTCATCATCTC; reverse: ATGCCTGCTTCACCACCTTCTTG). After an initial 1-min incubation at 96°C, the PCR was performed in two steps. The first step consisted of two cycles of 96°C for 1 min, 56°C for 4 min, and 70°C for 2 min. In initial experiments, products from cycles 20, 25, and 30 for each gene were analyzed by gel electrophoresis to insure that all amplifications were taking place in a linear manner.

Statistical Analysis

Results were analyzed by Student *t*-test, and differences from controls were considered significant at $P < 0.05$

RESULTS

Model of Decreased Intracellular K^+

In this study, we adopted a model, developed originally in lymphocytes [19, 29], that uncouples K^+ loss from the rest of the apoptotic cascade. When thymocytes are cultured in a hypotonic medium, they take on water and swell. This morphologic perturbation activates a regulatory volume decrease (RVD) response mechanism that actively pumps K^+ out of the cell. Loss of K^+ osmotically draws water out the cell, forcing it to shrink. The result at equilibrium is a cell

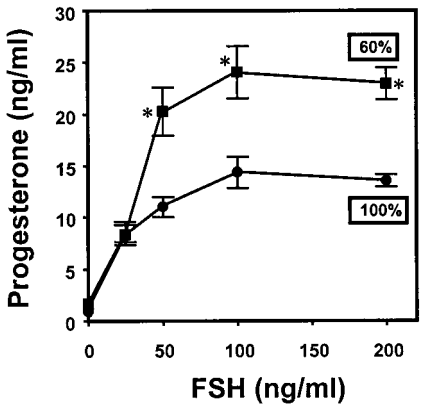

FIG. 4. FSH dose-response in 100% and 60% medium. Granulosa cells were cultured in medium diluted to 100% or 60% of normal for 4 h in the presence of increasing doses of FSH prior to analysis of progesterone levels. Data represent the mean ± SEM from three separate experiments (*$P < 0.05$ compared with cells cultured in 100% medium).

of near normal size with a significantly lowered resting $[K^+]_i$. To insure that a similar RVD response mechanism exists in granulosa cells, cells were cultured in McCoy 5a medium (100% hypotonicity) or in the same medium diluted with H_2O to 60% of normal. Preliminary studies indicated that 60% of normal hypotonicity represented the largest dilution from which the cells can recover without significant lysis. Although this dilution likely results in a cell with a higher intracellular K^+ than an apoptotic cell, it represents the best approximation currently available. Flow cytometric analysis of cell size indicated that granulosa cells cultured in 60% medium for 4 h retain a similar size distribution as cells cultured in 100% medium, with the exception of a minor population displaying a decreased forward scatter (Fig. 1A). This minor population represents a diminutive number of cells undergoing apoptosis at this time (Fig. 2). When the cells are loaded with the K^+ indicator dye PBFI-AM, a significant decrease in fluorescence is detected compared with cells cultured in 100% medium (Fig. 2B). Thus, granulosa cells possess an intact RVD mechanism, which we exploited to experimentally lower their resting $[K^+]_i$.

Reducing intracellular K^+ in thymocytes does not induce apoptosis but does increase a given cell's susceptibility to an apoptotic stimulus [19]. Placing granulosa cells in culture stimulates them to undergo apoptosis because of a withdrawal of in vivo survival factors, and thus culture in 60% medium would be expected to enhance this response. We next assessed the time course of apoptosis in granulosa cells cultured in 100% and 60% medium (Fig. 2). As expected, culture in 60% medium greatly enhanced the onset of apoptosis in these cells compared with cells cultured in 100% medium, as measured by the percentage of the population possessing a hypodiploid amount of DNA. However, a significant increase in cell death was not detected until 6 h, with only minor differences detected at 4 h. Together, these data (Figs. 1 and 2) indicate that granulosa cells cultured for 4 h in 60% medium possess a significantly reduced resting $[K^+]_i$ but have not yet initiated apoptosis to a significant degree. Thus, by examining cells under these parameters we can uncouple the effect of reduced intracellular K^+ from the majority of apoptotic events. All subsequent experiments were performed for 4 h.

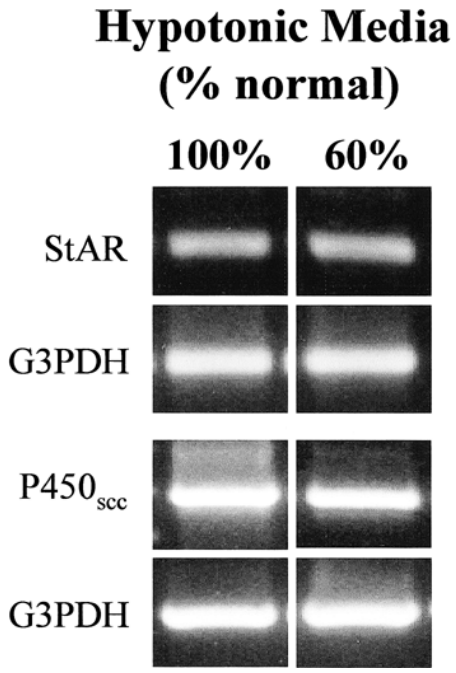

FIG. 5. Intracellular K^+ levels do not affect transcription of StAR and $P450_{scc}$ mRNA in FSH-stimulated granulosa cells cultured in diluted medium. Granulosa cells were cultured in 100% or 60% diluted medium for 4 h in the presence of 50 ng/ml FSH prior to analysis of mRNA levels via RT-PCR. PCR product was stained with ethidium bromide and run on a 1% agarose gel.

Hypotonic Medium Enhances FSH-Stimulated Progesterone Production but Not Estrogen Production

To assess the effects of hypotonic medium on steroidogenesis, granulosa cells were cultured in various dilutions of medium and stimulated with 50 ng/ml FSH for 4 h. Medium was then removed and assayed for progesterone and estrogen levels. Progesterone synthesis was increased at each dilution examined (Fig. 3), suggesting that decreased $[K^+]_i$ may promote the functional increase in progesterone production by dying granulosa cells, while estrogen production was not effected by dilution of the medium. In addition, the lack of an effect on estrogen synthesis provides further evidence that the cells are not undergoing significant amounts of apoptosis. If cells were dying in significant numbers, estrogen production would be diminished [4-7]. When cultured in 100% medium, both progesterone and estrogen were increased in FSH-stimulated cultures compared with unstimulated cultures, indicating intact and functional signalling pathways for each (data not shown).

Hypotonic Medium Enhances FSH-Stimulated but Not Basal Progesterone Production

We examined the effects of increasing levels of FSH on progesterone production by cells cultured in 100% and 60% medium. Dilution of the medium had no effect on progesterone synthesis in the absence of FSH (Fig. 4). Although cells cultured in both types of medium produced a dose-dependent response to FSH, maximal progesterone synthesis was enhanced in 60% medium.

Hypotonic Medium Does Not Increase mRNA Levels of StAR or $P450_{scc}$ in FSH-Stimulated Granulosa Cells

To explore the mechanism by which hypotonic medium exerts its effect, we examined a potential change in the mRNA level of two known rate-limiting components of the progesterone biosynthesis pathway, StAR and $P450_{scc}$ [4-7]. RT-PCR analysis did not reveal any change in the levels of StAR or $P450_{scc}$ mRNA within cells cultured in a diluted medium (60%) compared with those cultured in normal medium (100%) (Fig. 5).

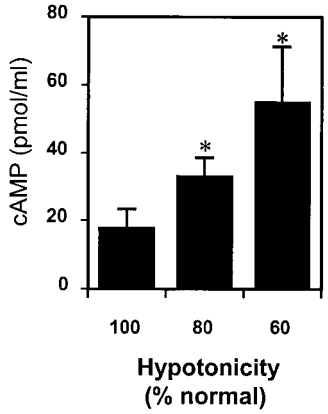

FIG. 6. Production of cAMP in FSH-stimulated granulosa cells cultured in media of increasing dilution. Granulosa cells were cultured in the presence of 50 ng/ml FSH and 0.5 mM IBMX for 4 h in medium diluted to 100%, 80%, or 60% of normal. Cells were pelleted and lysed in 0.1 N HCl prior to analysis of cAMP levels. Data represent the mean ± SEM from three separate experiments ($*P < 0.05$ compared with cells cultured in 100% medium).

Hypotonic Medium Enhances FSH-Stimulated cAMP Production

The inability of hypotonic medium to affect basal progesterone production suggests an effect on the FSH-signalling pathway. The FSH receptor signals through a G-protein to stimulate adenylate cyclase to produce cAMP. Therefore, we explored the effects of hypotonic medium on cAMP production. Cyclic AMP production was increased in a dilution-dependent manner (Fig. 6), suggesting that low intracellular K^+ may enhance an early step in the signalling pathway of FSH, in particular that leading to cAMP production.

DISCUSSION

Apoptotic cells extrude tremendous amounts of K^+ as they die, bringing the resting intracellular levels down to approximately 35 mM [18–20, 32]. This dramatic decrease is necessary for the activation of central enzyme systems such a caspases and nucleases, which are inactive at normal levels of K^+ (~140 mM) [18]. We explored a role for this loss of K^+ in regulating a unique feature of dying granulosa cells, the shift in steroidogenesis from estrogen to progesterone production. We adapted and validated in granulosa cells a model (originally developed in thymocytes [19, 29]) in which we can experimentally reduce $[K^+]_i$ by culturing a cell in hypotonic medium. Although the decrease in $[K^+]_i$ is not as extensive as that in an apoptotic cell, it does allow us to uncouple K^+ loss from the rest of the apoptotic cascade. Using this model, we found that FSH-stimulated progesterone production was significantly enhanced, although there were no effects on estrogen secretion or basal progesterone production. Cyclic AMP levels were also increased, suggesting the intriguing possibility that this response is mediated at the level of the FSH-signalling pathway. The increase in progesterone synthesis in dying gran-

ulosa cells represents a unique model in which dying cells perform a critical function en route to death, and the present study is the first to elucidate a mechanism underlying this change.

Although the increase in progesterone biosynthesis is a very striking feature of dying granulosa cells and ovarian follicular atresia, atretic follicles are also known to halt production of estrogen [4-7]. However, in the current studies hypotonic medium had no effect on estrogen production, suggesting that the apoptotic changes in estrogen and progesterone synthesis are regulated independently. These results support those of Terranova [5], who demonstrated in hamsters that the decrease in estrogen synthesis was a result of reduced androgen substrate produced by the theca cells and not a direct effect of the granulosa cells themselves. Because the cultures in these experiments contained an excess of androgens, it is not surprising that we did not see a change in estrogen biosynthesis. In contrast, investigators have more recently suggested that the onset of apoptosis has a direct effect on the potential of granulosa cells to produce estrogens [33] by reducing the mRNA transcript level of aromatase. Given the short time frame of the current studies, we cannot rule out an eventual effect of K^+ on aromatase mRNA levels. Unfortunately, those kinetic studies cannot be performed with the current model without a significant onset of apoptosis. The lack of an effect on estrogen synthesis provides evidence for specificity of the K^+ effects on progesterone synthesis and indicates that significant numbers of cells are not undergoing apoptosis. If cells were dying in significant numbers, estrogen production would be diminished [4-7].

Enzymes are known to be highly sensitive to the composition of the buffer in which they are active. This well-known fact led us to originally speculate that the increase in progesterone synthesis by cells cultured in hypotonic medium was the result of a direct effect on the steroidogenic enzymes, in particular $P450_{scc}$ or 3β-HSD. However, no effect on basal steroid production was seen in these steroidogenically active cells, so we focused our attention on the FSH-signalling pathway. One potential caveat in these studies is that the hypotonic medium simply promotes better FSH binding to its cognate receptor, leading to greater receptor occupation and an enhanced response at a submaximal dose of the gonadotropin. However, the dose-response studies demonstrated that maximal progesterone production is enhanced by hypotonic medium, suggesting a postreceptor target for this effect.

Analysis of cAMP levels suggests that low $[K^+]_i$ functions, at least in part, to enhance the FSH-signalling pathway. The exact mechanism by which this occurs (e.g., enhanced nucleotide exchange, direct effect on adenylate cyclase activity) is unknown and the subject of ongoing studies. In the present study, a direct effect of K^+ cannot be separated from the effect of decreased intracellular osmolality brought about by the lowered K^+ level. The effects of intracellular K^+ on apoptotic enzymes such as caspases and nucleases are not specific to K^+ and can be mimicked in vitro by any monovalent cation [18]. However, K^+ is of importance because it is the ion actually present at high concentrations inside cells. Thus, the findings in this study are a result of the decreased K^+ levels even though we cannot differentiate a direct effect from an effect of the decreased resting hypotonicity.

The downstream targets of cAMP may also be affected by the reduced ionic strength of the intracellular environment. For example, cAMP enhances steroidogenesis in part by upregulating synthesis of StAR [34, 35], a rate-limiting component of the pathway that functions to transport cholesterol into the mitochondria, and $P450_{scc}$, the first step in the steroidogenic pathway. However, with RT-PCR analysis we detected no change in the level of expression of mRNA encoding these molecules, suggesting that their activity may be modified posttranslationally. StAR in particular can be acutely regulated by phosphorylation and is a target for protein kinase A [36, 37]. Thus, low intracellular K^+ may alter the cAMP signalling pathway, suppressing its transcriptional effects while enhancing phosphorylation of StAR. The absence of a transcriptional effect may also result from a general shut down in new gene transcription/translation in preparation for death. In this case, the existence of a transcription-independent mechanism of enhancing FSH signalling would insure its functioning in the early stages of death.

These studies suggest for the first time that FSH stimulation is an important and necessary occurrence for dying granulosa cells to upregulate progesterone synthesis. In contrast, previous studies documented that gonadotropin receptor levels and gonadotropin binding decreases in dying granulosa cells [33, 38–42], although binding is not completely absent. This finding suggests an intriguing mechanism by which the granulosa cell limits the steroidogenic response. K^+ loss is a very early event in apoptosis and occurs rapidly [18–20, 43, 44], only later followed by activation of dismantling systems such as the effector caspases. Early loss of K^+ may greatly enhance a cell's progesterone production in response to circulating gonadotropins, but as apoptosis proceeds various systems intercede to reduce gonadotropin receptor levels and blunt the response. Eventually, the cell ceases all functions as it undergoes the final stages of death and is phagocytosed by neighboring cells or macrophages.

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