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Cyclic AMP-elevating Agents Promote Cumulus Cell Survival and Hyaluronan Matrix Stability, Thereby Prolonging the Time of Mouse Oocyte Fertilizability*[S]

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Cumulus cells sustain the development and fertilization of the mammalian oocyte. These cells are retained around the oocyte by a hyaluronan-rich extracellular matrix synthesized before ovulation, a process called cumulus cell-oocyte complex (COC) expansion. Hyaluronan release and dispersion of the cumulus cells progressively occur after ovulation, paralleling the decline of oocyte fertilization. We show here that, in mice, postovulatory changes of matrix are temporally correlated to cumulus cell death. Cumulus cell apoptosis and matrix disassembly also occurred in ovulated COCs cultured *in vitro*.C OCse xpanded *in vitro* with FSH or EGF underwent the same changes, whereas those expanded with 8-bromo-adenosine-3′,5′-cyclic monophosphate (8-Br-cAMP) maintained integrity for a longer time. It is noteworthy that 8-Br-cAMP treatment was also effective on ovulated COCs cultured *in vitro*,p rolongingt hev italityo ft he cumulus cells and the stability of the matrix from a few hours to >2 days. Stimulation of endogenous adenylate cyclase with forskolin or inhibition of phosphodiesterase with rolipram produced similar effects. The treatment with selective cAMP analogues suggests that the effects of cAMP elevation are exerted through an EPAC-independent, PKA type II-dependent signaling pathway, probably acting at the post-transcriptional level. Finally, overnight culture of ovulated COCs with 8-Br-cAMP significantly counteracted the decrease of fertilization rate, doubling the number of fertilized oocytes compared with control conditions. In conclusion, these studies suggest that cAMP-elevating agents prevent cumulus cell senescence and allow them to continue to exert beneficial effects on oocyte and sperm, thereby extending *in vitro* the time frame of oocyte fertilizability.

The formation of the antral cavity in the ovarian follicle separates most of the follicle cells from the oocyte with the exception of a small mass of follicle cells, named cumulus cells or cumulus oophorus, which remain closely associated with the oocyte, forming the cumulus cell-oocyte complex (COC).[2] These cells play a critical role in the regulation of meiotic resumption and acquisition of developmental competence by the oocyte during the time preceding ovulation (1–5). Reciprocally, the oocyte deeply influences gene expression in these cells by modulating their response to gonadotropins and paracrine factors synthesized by the cells of the follicle wall, namely mural granulosa cells (6). One of the most evident changes controlled by the oocyte in mouse cumulus before ovulation is the expansion process (*i.e.* the synthesis of an abundant extracellular matrix with unique physical properties) (7). This matrix is highly hydrated and incredibly extensible and viscous due to the elevated concentration of high molecular weight HA probably cross-linked by proteins, such as IαI, PTX3, and TSG6 (8–12). During this process, the connections among cumulus cells and between cumulus cells and oocyte are progressively lost, but the cells remain associated with the oocyte, being embedded in the expanded matrix. This oocyte envelope is essential for successful ovulation and fertilization. The viscoelasticity of the matrix allows the oocyte to wriggle out of the follicle and to be captured by the ciliary epithelium of the oviduct (13, 14). In addition, abnormal cumulus expansion impairs oocyte fertilization (7). In fact, this matrix can be easily crossed by the sperm, and its integral components, as well as soluble factors released by the cumulus cells, are involved in attracting the sperm toward the oocyte and in promoting capacitation and initiating acrosome reaction, processes required for successful fertilization (15, 16). It is well known that oocytes must be fertilized within a narrow window of time from ovulation. After this time, a series of ooplasmic modifications, collectively known as oocyte aging, rapidly occurs in the female gamete, diminishing its fertilizability and embryo developmental potential (17, 18). Delayed fertilization of the ovulated oocytes results in early pregnancy loss and increased offspring morbidity in rodents and appears to increase the risk of abortion in humans (19–21). A reduction in meiotic promoting factor, which regulates the exit from Met II block, occurs in the mouse oocyte as early as 6 h after ovulation. Moreover, disorganization of cortical actin cytoskeleton and displacement and instability of the spindle are clearly apparent after 12 h of staying in the oviduct, accounting for the increased incidence of scattering of chromosomes and cytoplasm fragmentation upon fertilization that is a

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[S] This article contains supplemental Movies S1–S4.

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[2] The abbreviations used are: COC, cumulus cell-oocyte complex; HA, hyaluronan; hCG, human chorionic gonadotropin; 8-Br-cAMP, 8-bromo-adenosine-3′,5′-cyclic monophosphate; dbcAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate; 8-AHA-cAMP, 8-(6-aminohexylamino)adenosine- 3′,5′-cyclic monophosphate; 6-Mb-cAMP, N^6-monobutyryladenosine-3′,5′- cyclic monophosphate; 8-pCPT-2′-O-Me-cAMP, 8-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate; PMSG, pregnant mares' serum gonadotropin.

prelude to embryonic aneuploidy (17, 18). Interestingly, a progressive reduction in cumulus cell mass parallels the aging of the enclosed oocyte, leading almost to oocyte denudation in ~15 h (*i.e.* about 28 h after an ovulatory dose of human chorionic gonadotropin (hCG)) (22). Metabolic labeling of newly synthesized HA by COCs induced to expand *in vitro* with FSH allowed the determination that disassembly of the viscoelastic matrix begins 3–4 h after the completion of expansion and continues thereafter, promoting the shedding of cumulus cells (23, 24). The HA was released from the matrix into the medium without any significant variation in size (23), suggesting that the disassembly of the matrix is not dependent on cleavage of this polymer but rather on degradation of proteins involved in its organization. Degeneration of cumulus cells has been described in mouse postovulatory COCs (25) and apoptosis signature has been revealed in rat COCs after a prolonged staying in the oviduct (26). However, a precise estimate of the functional life of cumulus cells and its correlation with cumulus matrix degradation and oocyte aging is missing. In view of the pressing need to improve the conditions for promoting and preserving the quality of the oocytes during their culture and handling in assisted reproduction programs, we performed a systematic study on temporal patterns of cumulus cell apoptosis and dispersion in ovulated COC and in COC expanded *in vitro* in order to identify factors regulating these processes and to determine the impact they might have on the fertile life of the oocyte.

Experimental Procedures

Materials—Pregnantm ares's erumg onadotropin(PMSG) and hCG were purchased from Intervet (Boxmeer, The Netherlands). Highly purified rat FSH I-8 was kindly provided by the NIDDK and the National Hormone and Pituitary Program, National Institutes of Health (Bethesda, MD). Epidermal growth factor (EGF), cycloheximide, UO126, and *Streptomyces* hyaluronidase were purchased from Calbiochem. Transforming growth factor β (TGFβ) was obtained from R&D System. Minimal essential medium, fetal calf serum (FCS), gentamycin, and HEPES buffer were obtained from Gibco, Invitrogen. Mineral oil, L-glutamine, sodium pyruvate, 8-bromo-adenosine-3′,5′-cyclic monophosphate (8-Br-cAMP), dbcAMP, 8-AHA-cAMP, 6-Mb-cAMP, forskolin, H89, LY294002, and wortmannin were from Sigma. 8-pCPT-2′-O-Me-cAMP was from Biolog. Sephadex G50 was from Amersham Biosciences. [^{3}H]Glucosamine was obtained from PerkinElmer Life Sciences. The *in situ* cell death detection kit was purchased from Roche Applied Science.

Animals—Immature2 1–22-day-oldf emaleS wissC D1m ice were used for all experiments. Animals were primed by intraperitoneal injection of PMSG (5 IU) to promote the formation of multiple antral follicles and, 44–48 h later, received an intraperitoneal injection of hCG (5 IU) to induce ovulation. All experiments were approved by the institutional animal care and use committee and carried out according to Italian and European regulations (D.L.vo 116/92; C.E. 609/86).

Induction and Culture of in Vitro Expanded COCs—Ovaries were dissected from mice injected 44–48 h earlier with PMSG and transferred to minimal essential medium containing 25 mM Hepes, 50 μg/ml gentamycin, 0.3 mM sodium pyruvate, and 1 mg/ml BSA. COCs were isolated under the microscope by puncturing the larger follicles with a needle. For each culture, 10 compact COCs were collected by a glass micropipette and transferred to a 20-μl drop of culture medium covered with dimethylpolysiloxane to prevent evaporation. The basal culture medium was minimal essential medium supplemented with 5% FCS, 3 mM glutamine, 0.3 mM sodium pyruvate, and 50 ng/ml gentamycin. Expansion was induced by adding to the medium either 20 ng/ml FSH, 1 ng/ml EGF, or 1 mM 8-Br-cAMP, and the cells were cultured at 37 °C, 5% CO_2. Full expansion was achieved at 15 h of culture, and disassembly of the expanded matrix and cumulus cell apoptosis were investigated thereafter at the times indicated under "Results." When the effect of 8-Br-cAMP on FSH- or EGF-expanded COCs was studied, 2 μl of medium was removed from the 20-μl drop at 15 h of culture and replaced with 2 μl of 10 mM 8-Br-cAMP to reach a final concentration of 1 mM. The same procedure was used to study the effect of H89, LY294002, wortmannin, and UO126 on 8-Br-cAMP action. In this case, the inhibitors were added 1 h before (14 h of culture with FSH or EGF) 8-Br-cAMP. Cultures of isolated cumulus cells were performed by mechanical dissociation of 10 COCs in a 20-μl culture medium drop and removal of the oocytes, as described (27). Expansion was induced with 100 ng/ml FSH and 5 ng/ml TGFβ.

Isolation and Culture of Ovulated COCs—Ins tudieso fa poptosis *in vivo*,a nimalsw eres acrificeda t1 3h (timeo fo vulation), 20 h, or 24 h after hCG injection. Oviducts were dissected and transferred to HEPES-buffered medium (see above). Ampullae were opened with a needle, and the ovulated COCs were immediately processed. To study apoptosis in ovulated COCs cultured *in vitro*,C OCso btainedf romt hreeo viductso f three different mice were cultured in 500 μl of minimal essential medium supplemented with 1 mg/ml BSA, 3 mM glutamine, 0.3 mM sodium pyruvate, 50 ng/ml gentamycin and treated with 1 mM 8-Br-cAMP or other compounds reported under "Results" at 37 °C, 5% CO_2 for the time indicated. When the effect of H89, LY294002, wortmannin, and UO126 on 8-Br-cAMP action was studied, the inhibitors were added at the beginning of culture, and 8-Br-cAMP was added 1 h later. The dissociation of the matrix was assessed by morphological evaluation and expressed in arbitrary units from 0 (intact COC) to 4 (denuded oocyte).

In Vitro Fertilization—COCsw erec ollecteda t1 3h a fter hCG and cultured for 14 h in 500 μl of basal medium containing 5% FCS with or without 1 mM 8-Br-cAMP. To normalize biological variability, the COCs collected from the oviducts of each animal were equally distributed in the two experimental culture conditions. Oocytes denuded of cumulus cells were obtained from COCs isolated at 13 h after hCG by digesting the matrix with 30 units/ml testicular hyaluronidase for 5 min at room temperature. After dispersion of cumulus cells, denuded oocytes were collected and cultured in the same conditions and for the same time as COCs.

Cumulus cell oocyte complexes and denuded oocytes either freshly isolated (0 h of culture) or cultured *in vitro* for 14 h were transferred to a 100-μl drop of Whitten's medium supplemented with 15 mg/ml BSA. Spermatozoa were obtained from the epididymis of CD1-proven breeders and were capacitated

(2 h) in the same medium used for fertilization. Subsequently, 10 μl of a sperm suspension containing 5–10 × 10^6 spermatozoa/ml was added to oocyte cultures. After 6 h, the oocytes were observed via the interference-contrast microscope to determine the fraction undergoing fragmentation and the fraction normally fertilized, by the identification of the two pronuclei. The development of the embryo at the two-cell stage was assessed at 24 h after insemination.

Qualitative and Quantitative Analysis of Apoptosis—COCs were fixed for 30 min at room temperature with 4% paraformaldehyde in PBS (pH 7.4). After washing with PBS containing 1 mg/ml BSA, COCs were processed for TUNEL staining. Cells were permeabilized for 2 min in 0.1% Triton X-100 in PBS. After a wash with PBS containing 1 mg/ml BSA, COCs were incubated for 60 min at 37 °C with TUNEL reaction mixture, according to the manufacturer's instructions. A negative control was included by incubating COCs in TUNEL reaction mix without TdT. To visualize chromatin, COCs were counterstained with 5 μg/ml Hoechst 33258 for 5 min and washed extensively with PBS containing 1 mg/ml BSA. COCs were individually transferred in a small volume on a slide and gently pressed under a coverslip until cumulus cells, dispersed in the tridimensional structure of the matrix, lay on the same focal plane. Cells were examined by an Olympus Axioplan 2 microscope in fluorescent mode. Cells showing a homogeneous and moderate chromatin staining by Hoechst 33258 throughout the entire nucleus resulted in negative TUNEL staining and therefore were considered healthy cells. On the contrary, cells with nuclei containing chromatin deeply stained with Hoechst 33258, either merged into masses aligned with the nuclear membrane, shrunken into a single mass, or fragmented into multiple masses that occurred in clusters immediately adjacent to one another, resulted in TUNEL-positive staining and were therefore defined as apoptotic cells. Pilot experiments showed that quantitative evaluation of apoptosis performed by nuclear morphology matched that made by TUNEL staining. Therefore, in the following experiments, COCs were stained with Hoechst 33258, and cells with healthy and apoptotic features were counted in six random fields for each COC through a ×400 magnification in fluorescent mode. The percentage of apoptotic cells was calculated for each COC. Between 7 and 10 COCs for each sample were analyzed in each experiment.

Quantitative Analysis of HA Matrix Disassembly—In order to evaluate the distribution of HA between cell matrix and medium compartments during matrix disassembly, 10 COCs were cultured in a 20-μl drop of medium in the presence of FSH, EGF, or 8-Br-cAMP (see above) and with [^{3}H]glucosamine (100 μCi/ml). Cultures were analyzed at 15 h, when full expansion is achieved, and at later times, as indicated under "Results." At the end of culture, medium and cell matrix were processed separately, and the percentage of HA in the two compartments was assessed as described previously (28). Briefly, the incubation medium was aspirated, and the medium and matrix fractions were separately treated with 20 μl of a papain solution (750 mIU final activity) for 1 h a t6 5° C.T heextraction was completed by adding 1 volume of 8 M guanidine HCl containing 4% (w/v) Triton X-100. Each extract was heated at 100 °C for 3 min to inactivate the papain and diluted to 500 μl by adding 0.1 M Tris, 0.1 M sodium acetate, pH 7.3, followed by elution on a column of Sephadex G50 (2-ml bed volume) equilibrated with 0.1 M Tris, 0.1 M sodium acetate, and 0.5% Triton X-100, pH 7.3. The excluded volume, containing the labeled macromolecules, was recovered and counted. Each sample was then digested with 1 IU of *Streptomyces* hyaluronidase (Calbiochem) for 2 h a t 37 °C and then eluted on a column of Sephadex G-50 (8-ml bed volume). The excluded and the included fractions were counted to determine the HA proportion of the total radiolabeled molecules.

To compare the rate and time course of HA synthesis in different conditions, the mass of HA synthesized was determined (in hexosamine equivalents) at different times of culture from 3 to 40 h, as described previously (8). Briefly, COCs were stimulated in the presence of [^{35}S]sulfate (~60 μCi/ml) and [^{3}H]glucosamine (100 μCi/ml), and the specific activity of the UDP-*N*-acetylhexosamine pools was calculated from the ratio of ^{3}H to ^{35}S in the proteoglycan 4S disaccharides.

Morphological Analysis of Matrix Elasticity—COCs were transferred in a 20-μl drop of medium. Movies were captured during aspiration of the medium (*i.e.* under the pressure exerted by the increase of superficial tension), and during the release back of the medium (*i.e.* the decrease of the applied force). A Nikon Eclipse Ti microscope using a ×10, numerical aperture 0.25 Nikon PLAN objective and NIS Elements imaging software were used.

Statistical Analysis—Thed atar epresentt hem ean ± S.E. of at least three independent experiments. Statistical significance of the difference between two treatments was analyzed by Student's *t* test, whereas one-way analysis of variance with the addition of Turkey's test was used for multiple comparisons. A *p* value of <0.05 was considered significant.

Results

Cumulus Cell Apoptosis Parallels Matrix Disassembly in Ovulated COCs—Followingh CGi njectioni ntoP MSG-primed mice, compact COCs undergo volumetric expansion for the deposition of an abundant HA-enriched extracellular matrix. At 13 h after hCG, fully expanded COCs are released from the ovarian follicles and captured by the oviducts. A progressive reduction in cumulus cell mass occurs in the oviduct during the following time leading almost to oocyte denudation in ~11–15 h (24–28 h after hCG) (Fig. 1*A*). Nuclear morphology of cumulus cells was examined at different times during the postovulatory period by Hoechst 33258 staining (Fig. 1*B*). At ovulation (13 h after hCG), almost all cumulus cells showed intact nuclei with low and quite homogeneous chromatin staining, but 7 and 11 h later (at 20 and 24 h after hCG, respectively), a large number of cells with highly condensed chromatin, either shrunken into a single mass or fragmented into multiple masses, was observed. TUNEL staining clearly revealed that cells with the above mentioned morphological features contained fragmented DNA (Fig. 1*B*). Then, for a quantitative evaluation of apoptosis, chromatin staining was performed, and the number of apoptotic cells was counted and expressed as a percentage of the total number of cells/COC (Fig. 1*C*). Cumulus cell-oocyte complexes isolated from the ovary 4 h before ovulation (9 h after hCG) showed almost no sign of apoptosis (0.9 ± 0.05%),

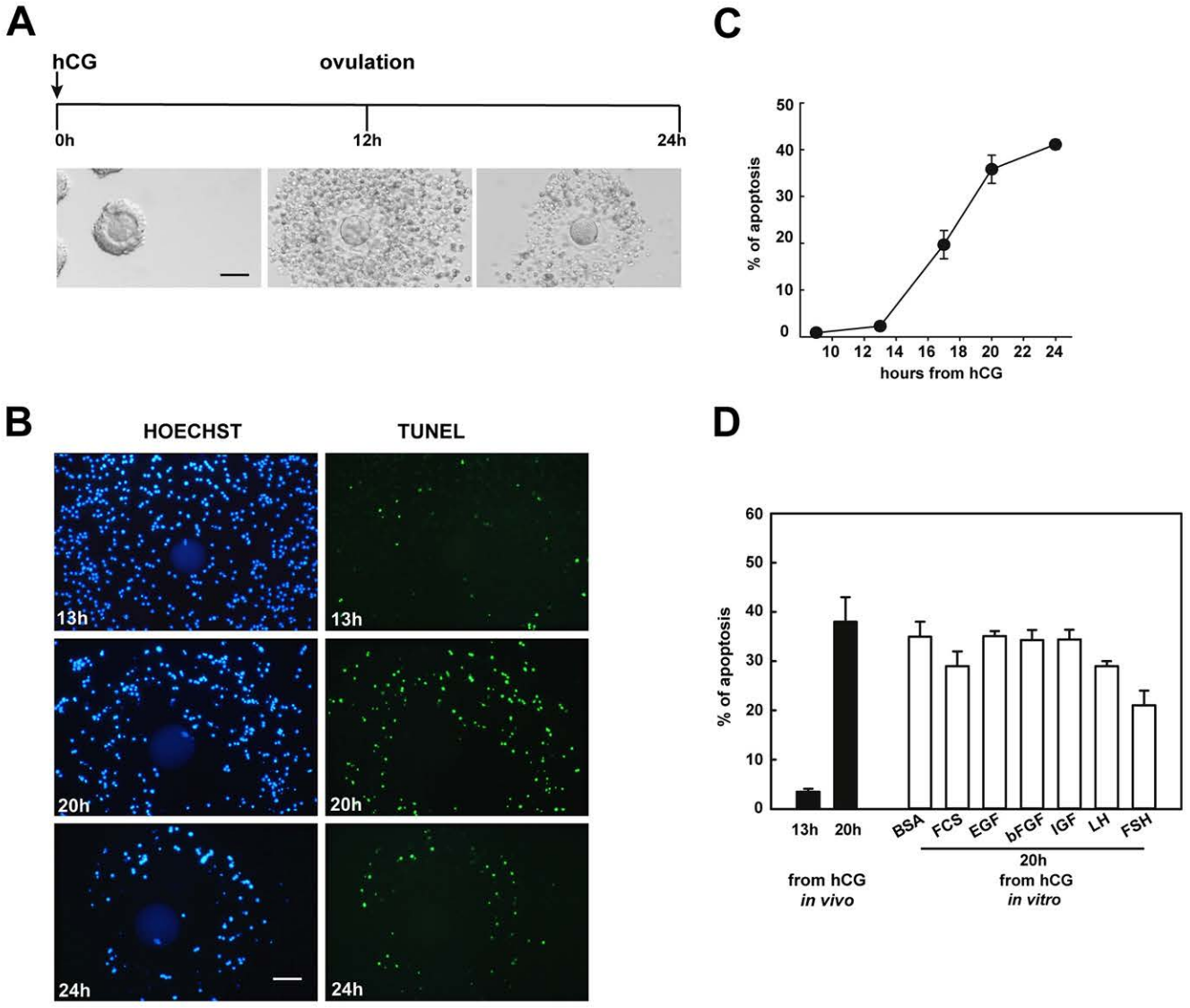

FIGURE 1. **Cumulus cells undergo apoptosis after ovulation.** Mice were injected with PMSG to promote the formation of antral follicles and, after 48 h, were injected with hCG to induce ovulation. COCs were isolated at different times after the administration of hCG. *A*, morphological changes of COCs before and after ovulation. Light micrographs of compact (0 h), expanded (12 h), and dissociated (24 h) COCs isolated from the ovary and from the oviducts at the indicated hours after hCG injection. *Bar*, 100 μm. *B*, representative images of apoptotic staining at fluorescent microscopy. Fixed COCs were individually transferred on a slide and gently pressed under a coverslip until cumulus cells, scattered in the muco-elastic matrix, lay on the same focal plane. Nuclear morphology was examined in ovulated COCs by Hoechst 33258 staining (*blue*), and DNA fragmentation was assessed by TUNEL (*green*). *Bar*, 100 μm. *C*, time course and quantitative analysis of apoptosis. Apoptosis was determined by assessment of nuclear morphology using Hoechst 33258, as reported under "Experimental Procedures." Total and apoptotic cells were counted in six random fields of each COC through a ×400 magnification in fluorescent mode, and the percentage of apoptotic cells was calculated for each COC. 7–10 COCs for each experimental sample were analyzed. The data represent the mean ± S.E. (*error bars*) o fa t least three independent experiments. *D*, influence of serum, growth factors, and gonadotropins on apoptosis of *in vitro* cultured ovulated COCs. Ovulated COCs were collected from the oviducts at 13 and 20 h after the administration of hCG (*solidb ars*) or collected at 13 h and cultured for 7 h (total of 20 h after hormone stimulation) (*emptyb ars*)w ith1 m g/mlB SAa loneo rw ith5 %F CS,1 0n g/mlE GF,3 0n g/mlb asicF GF(*bFGF*), 100 ng/ml insulin-like growth factor (*IGF*), 500 ng/ml LH, or 100 ng/ml FSH. The values are expressed as a percentage of apoptotic cells and represent the mean ± S.E. of at least three independent experiments. None of the treatments produced a rate of apoptosis significantly different from the control (BSA) ($p > 0.05$).

and those freshly ovulated (13 h after hCG) had very few apoptotic cells (2.3 ± 0.5%). The percentage of apoptotic cells abruptly increased thereafter, ~8- and 15-fold at 4 and 7 h after ovulation, respectively (17 and 20 h after hCG), reaching a value of 35.8 ± 3%. The apoptotic rate did not apparently increase further during the following 4 h (*i.e.* at 24 h after hCG). However, at this later time, the number of cells surrounding the oocyte was extremely reduced, and much scattered cellular debris containing a variable amount of condensed chromatin was present (Fig. 1*B*). For this reason, 20 h after hCG was the time chosen to investigate the apoptotic process in the following experiments.

To determine whether cumulus cell apoptosis was induced by the oviductal environment, COCs were collected at 13 h after hCG and cultured *in vitro*. The number of apoptotic cells/COC was then evaluated after 7 h of culture and compared with that observed in COCs retrieved from the oviducts 7 h after ovulation (*i.e.* at 20 h after hCG). The results showed that the percentage of apoptotic cells significantly increased during *in vitro* culture, reaching the same value observed in COCs main-

tained for the same time in the oviduct (Fig. 1*D*). Therefore, it seems that cumulus cell death is not due to detrimental effects by the oviduct.

A series of studies highlighted the essential role of endocrine and paracrine factors in sustaining mural granulosa cell survival in the ovarian follicles (29). It has been reported that mural granulosa cells cultured *in vitro* undergo apoptosis unless serum or growth factors, including EGF, basic FGF, insulin-like growth factor, or gonadotropins were added to the cultures. To investigate whether the onset of cumulus cell apoptosis after ovulation could be due to the deprivation of follicle survival factors, ovulated COCs were cultured for 7 h with the factors mentioned above (Fig. 1*C*). As shown in Fig. 1*D*, neither serum nor any of the growth factors tested were effective in preventing spontaneous onset of apoptosis by ovulated cumulus cells. Luteinizing hormone also failed in suppressing cumulus cell death, and FSH slightly inhibited the process, although the difference from control (BSA) did not reach statistical significance ($p = 0.059$).

According to previous findings (24), COC matrix became loose during culture in all conditions mentioned above, and apoptotic and healthy cells were progressively released into the medium. The healthy cells individually settled on the dish, and those that formed aggregates or adhered to the plastic, in the absence and in the presence of serum, respectively, remained viable for several days (data not shown).

Cumulus Cell Apoptosis and Matrix Disassembly in COC Matured in Vitro—Tod eterminew hetherc umulusc ella poptosis also occurs in COC matured *in vitro*,c ompactC OCsw ere isolated from PMSG-primed follicles and cultured in the presence of 5% FCS with either FSH or EGF or the membrane-permeable cAMP analogue 8-Br-cAMP. These factors were shown previously to stimulate *in vitro* COC expansion, which marks the final maturation of the cumulus. Metabolic labeling showed that, whatever the factor used, the amount of newly synthesized HA increased up to 15 h of culture and remained constant thereafter (Fig. 2*A*). Thus, the time course of apoptosis was evaluated in each condition starting from 15 h of culture (Fig. 2*B*), when HA synthesis ceased and full expansion was achieved. The apoptotic rate in COCs cultured in the presence of FSH was low at the end of the expansion process, ranging between 3 and 4%, and progressively increased after 18 h of culture, reaching a value of ~20% at 24 h (9 h after completion of expansion), mimicking the *in vivo* condition. Shedding of apoptotic and healthy cumulus cells from the complex to the culture medium occurred during the culture time leading to oocyte denudation (Fig. 2*D*). In agreement, metabolic labeling of HA synthesized during *in vitro* expansion showed that 80–90% of HA was organized in the matrix at 15 h and that a progressive HA release into the medium occurred afterward in parallel to the increase of the apoptotic rate (Fig. 2*C*). Interestingly, the number of apoptotic cells in EGF-stimulated COCs was significantly higher than in FSH-treated COCs at the completion of expansion (Fig. 2*B*) ($p < 0.001$). Nonetheless, the apoptotic rate abruptly increased between 18 and 24 h of culture, coincident with HA release and cumulus dispersion, similar to what was observed for the FSH-treated COCs (Fig. 2, *B–D*). It is noteworthy that COCs cultured in the presence of

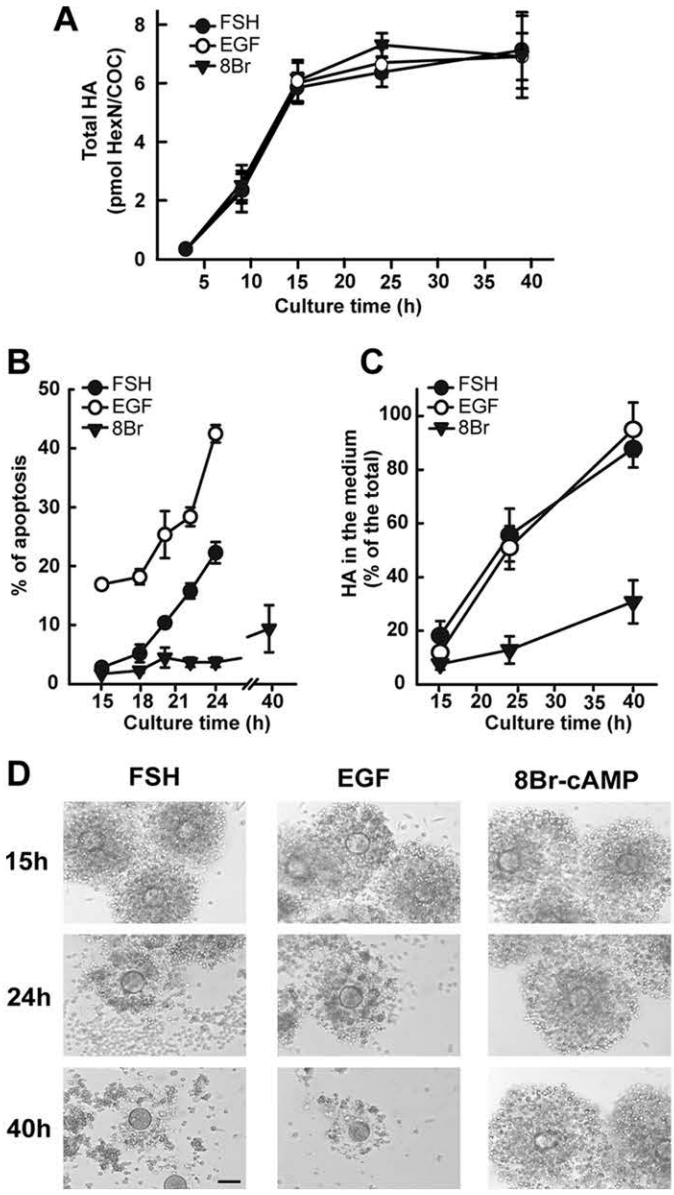

FIGURE 2. **Temporal pattern of apoptosis and matrix disassembly in COC matured *in vitro*.** Compact COCs were collected from PMSG-primed mice and induced to expand *in vitro* with 20 ng/ml FSH, 1 ng/ml EGF, or 1 mM 8-Br-cAMP in the presence of 5% FCS. *A*, time course of HA synthesis. The amount of HA (in hexosamine equivalents) was determined, as described under "Experimental Procedures," at different culture times from 3 to 40 h. The values represent the mean ± S.E. (*error bars*) of three independent experiments. *B*, COCs were fixed at the time of cumulus expansion completion (15 h) and at different times following, and the apoptotic rate was evaluated as described in the legend to Fig. 1. The values are expressed as a percentage of apoptotic cells and represent the mean ± S.E. of at least three independent experiments. *C* and *D*, disassembly of the HA-rich matrix synthesized during expansion was assessed at 24 and 40 h of culture by morphological analysis at the inverted microscope (*D*) (*bar*, 100 μm) and quantified by calculating the proportion of the total HA that was released from the matrix in the medium, following metabolic labeling (*C*), as described under "Experimental Procedures." The values represent the mean ± S.E. of five independent experiments.

8-Br-cAMP (or dbcAMP; data not shown), as with FSH, showed a low number of apoptotic cells at 15 h, but, conversely to what was observed with the gonadotropin, the apoptotic rate did not increase at 24 h. Even at 40 h of culture (25 h after completion of expansion), the apoptotic rate did not exceed

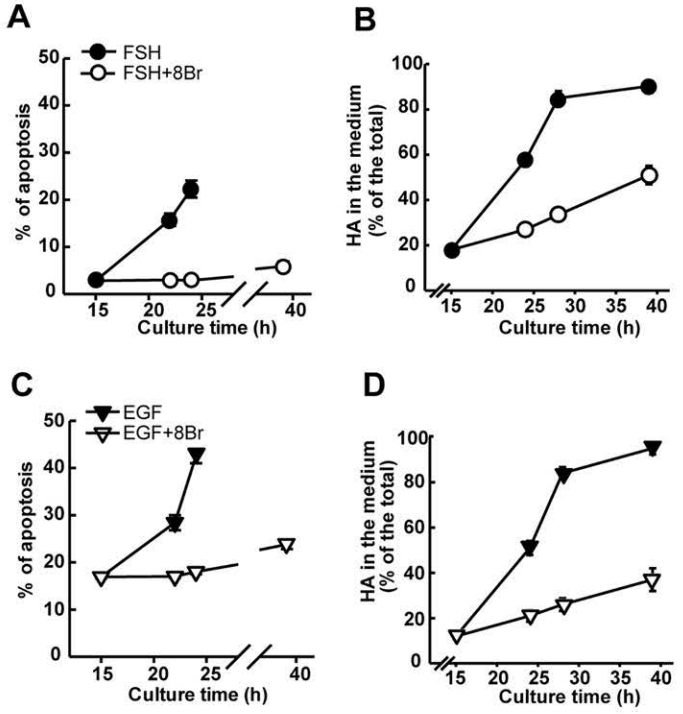

FIGURE 3. **Onset of spontaneous apoptosis and matrix disassembly in FSH- and EGF-stimulated COCs are counteracted by 8-Br-cAMP at an early pre-apoptotic stage.** Compact COCs were cultured with 20 ng/ml FSH (*A* and *B*) or 1 ng/ml EGF (*C* and *D*) in the presence of 5% FCS. In some cultures, 8-Br-cAMP was added at 15 h at the final concentration of 1 mM. The apoptotic rate (*A* and *C*) and the HA release (*B* and *D*) were analyzed in all conditions at 15 h and at different times following. For HA metabolic labeling, [^{3}H]glucosamine was added at the beginning of culture. The values are mean ± S.E. (*error bars*) of at least three independent experiments.

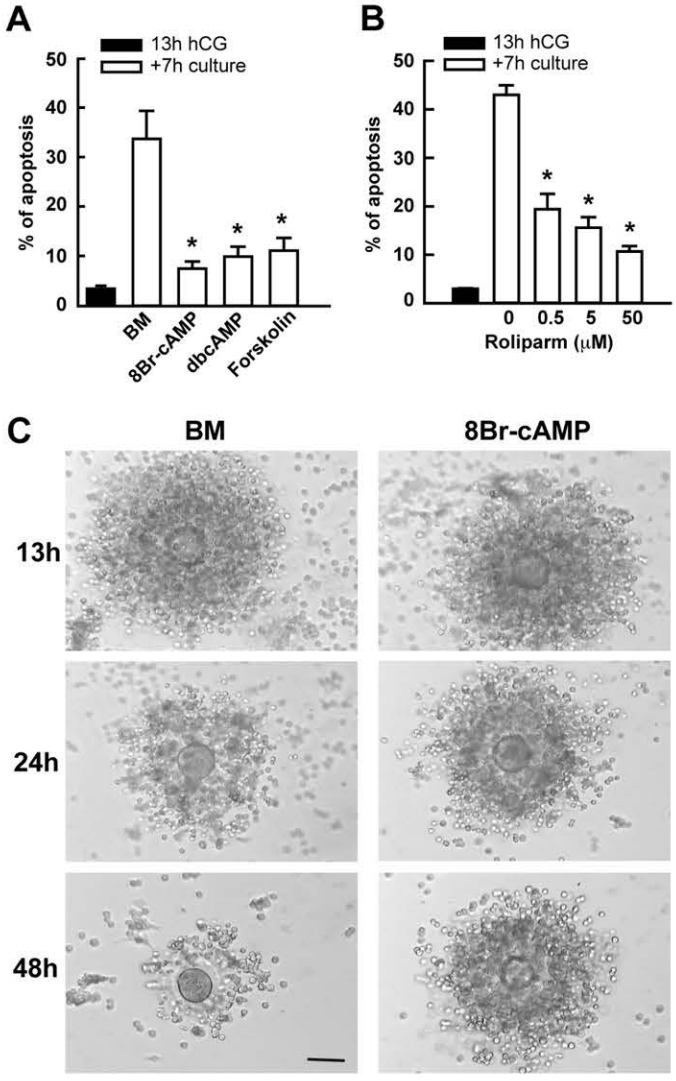

FIGURE 4. **Elevation of cAMP level prolongs *in vitro* cell viability and integrity of ovulated COCs.** Ovulated COCs were collected at 13 h after hCG, and the apoptotic rate was evaluated at 7 h o fculture(total2 0h a fterh CG)i n the absence (basal medium (*BM*)) or in the presence of 1 mM 8-Br-cAMP, 1 mM dbcAMP, or 10 μM forskolin (*A*) or with different concentrations of rolipram (*B*). The values are the mean ± S.E. (*errorb ars*) of at least three independent experiments. *, statistically significant difference from control (*BM* or *0*). *C*, representative micrographs of ovulated COCs collected at 13 h after hCG and cultured in the presence of 8-Br-cAMP for 11 and 35 h (*i.e.* for a total of 24 and 48 h after hCG). Micrographs were taken by an inverted microscope. *Bar*, 100 μm.

10%. In addition, whereas in FSH-stimulated COCs, the elastic matrix, formed within the first 15 h of culture (supplemental Movie S1),w asc ompletelyd isassembleda t4 0h a ndt hec umulus cells dissociated from the oocyte (supplemental Movie S2), COCs cultured for 40 h with 8-Br-cAMP had a large number of cells still surrounding the oocytes (Fig. 2*D*) and showed the same ability as at 15 h of culture to stretch under compression and to resume their shape after force release (supplemental Movies S3 and S4), suggesting that the structure and the mucoelastic properties of the matrix were preserved by this cAMP analogue. In agreement, no significant amount of HA was shed into the medium between 15 and 24 h of culture, and only 25% HA was released during the following 16 h (Fig. 2*C*).

Elevation of cAMP Levels Prevents Apoptosis and Matrix Disassembly in COCs Matured in Vitro and in Vivo—Theo bservation that apoptosis and matrix disassembly were abrogated in the presence of 8-Br-cAMP prompted us to assess whether COCs expanded in response to FSH or EGF could be rescued by 8-Br-cAMP. Compact COCs were cultured in the presence of FSH or EGF for 15 h. At that time (*i.e.* before the beginning of the degradation process), 8-Br-cAMP was added to the culture medium. The results showed that the cAMP analogue was able to prevent apoptosis in FSH-stimulated COCs and significantly delay the dissociation of the matrix up to 40 h of culture (Fig. 3, *A* and *B*). Similar results were obtained when COCs were expanded with EGF (Fig. 3, *C* and *D*). Indeed, although EGF-stimulated COCs showed an elevated apoptotic rate at 15 h, the addition of 8-Br-cAMP prevented the progression of this process as well.

Then we investigated whether cell viability and matrix stability of *in vivo* matured COC could be prolonged in culture. For this purpose, COCs were collected from the oviducts soon after ovulation (13 h after hCG) and cultured with or without 8-Br-cAMP or dbcAMP for 7 h. The results show that both cAMP analogues reduced by 70 – 80% the apoptotic rate (Fig. 4*A*). The same result was obtained by culturing COCs with forskolin, which, activating adenylate cyclase, increases the endogenous intracellular cAMP level. In addition, rolipram, a specific inhibitor of phosphodiesterase 4 that is mainly responsible for cAMP degradation in cumulus cells (30), attenuated apoptosis in a dose-dependent manner (Fig. 4*B*). Notably, the treatment with

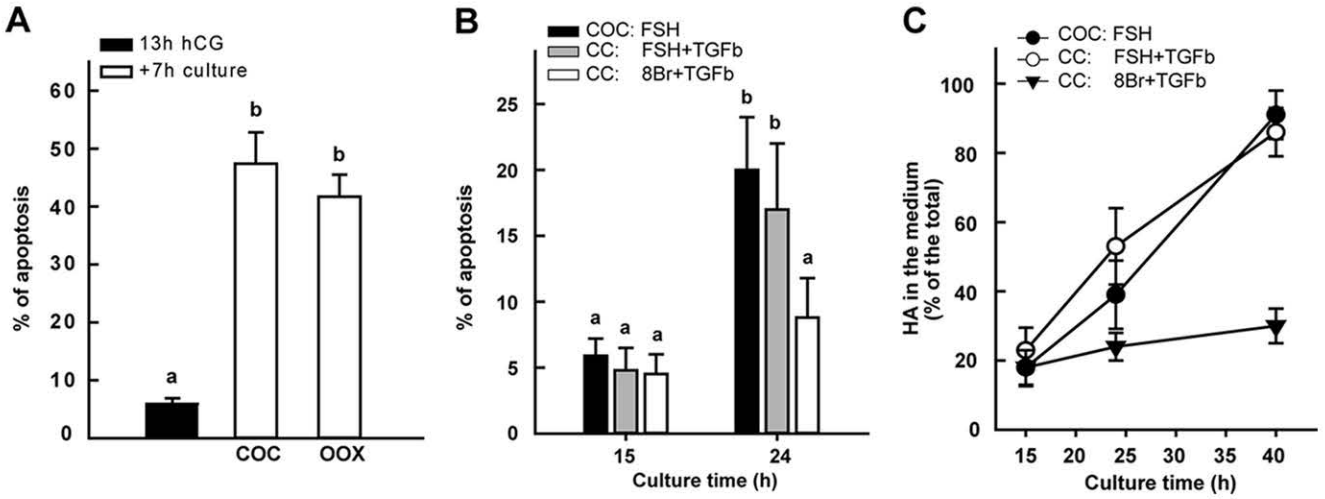

FIGURE 5. **The oocyte does not influence the pattern of apoptosis and matrix disassembly in COCs after ovulation.** *A*, ovulated COCs were collected at 13 h after hCG, and the enclosed oocyte was punctured with a needle under the microscope. The resulting oocytectomized complexes (*OOX*) consisted of a zona pellucida surrounded by the expanded cumulus. Intact COCs and oocytectomized complexes were cultured for 7 h (total 20 h after hCG) with 1 mg/ml BSA. The values are mean ± S.E. (*error bars*)o ff oure xperiments. *B* and *C*, compact COCs were collected 48 h after PMSG injection, and some of them were mechanically dissected, and the oocyte was removed. To induce the synthesis of HA-rich expanded matrix, COCs were cultured with 5% FCS, 20 ng/ml FSH, or 1 mM 8-Br-cAMP, and cumulus cells (*CCs*) were cultured in the same conditions plus 5 ng/ml TGFβ, which mimics the oocyte action on cumulus expansion (33). Apoptotic rate (*B*) and HA release (*C*) were analyzed at the indicated culture times. The values are the mean ± S.E. of three independent experiments. In *A* and *B*, multiple comparison analysis (one-way analysis of variance) was performed, and significant differences are indicated by *different superscript letters* ($p < 0.05$).

8-Br-cAMP maintained the integrity of ovulated COCs beyond 48 h after hCG injection (*i.e.* for >1.5 days of culture) (Fig. 4*C*).

Previous studies have shown that oocyte secreted factors play critical roles in the regulation of cumulus cell functions before ovulation, including the promotion of cumulus cell survival (31) and cumulus matrix deposition (27, 32). We then assessed whether the oocyte might influence the grade and the time course of cumulus cell apoptosis and matrix remodeling after ovulation. To this purpose, ovulated COCs were deprived of oocytes (oocytectomized complexes) and cultured *in vitro*. Analysis at 7 h o fculture showed no difference in the apoptotic rate between cumuli cultured with and without the enclosed oocyte (Fig. 5*A*) ($p = 0.097$). Similar results were obtained with isolated cumulus cells expanded *in vitro*.C ompactC OCsw ere mechanically dissociated, and oocyte was discarded. Isolated cumulus cells were then stimulated with FSH in the presence of TGFβ, a growth factor able to substitute for the oocyte in promoting expansion (33). Apoptotic rate as well as the temporal pattern of HA release from the expanded matrix were similar in cumulus cells cultured with (intact COC) and without oocytes (*CC*)(Fig.5 , *B* and *C*). Notably, 8-Br-cAMP inhibited cumulus cell apoptosis and HA release also in the absence of oocytes, indicating that the beneficial effect of 8-Br-cAMP is direct on cumulus cells and not mediated by the germ cell (Fig. 5, *B* and *C*).

The Effect of 8-Br-cAMP Is Exerted through PKA Activation—Mechanisms for the response to cAMP can involve either the protein kinase A (PKA), also known as cAMP-dependent protein kinase, or EPAC (exchange protein directly activated by cAMP), which is a guanidine nucleotide exchange factor for the small GTPases Rap1 and Rap2 (34). To discriminate between the two possibilities, we used cAMP analogues with higher affinity for one or the other signaling molecule. We found that the cAMP analogue 8-pCPT-2′-*O*-Me-cAMP, capable of selective EPAC activation (35), was unable to maintain the integrity of COCs at any of the concentrations tested up to 1 mM (Fig. 6).

Two major isozymes of PKA, type I and type II, were found, which differ in the regulatory subunit (R). In each subunit R, there are two cAMP binding sites, site 1 and site 2, and when both sites are occupied by cAMP, a drastic reduction in the affinity of R to the catalytic subunit occurs, leading to the dissociation of active catalytic subunits. The two types of holoenzymes can be selectively activated by the synergistic action of pairs of specific cAMP analogues that at low concentrations individually bind to site 1 and site 2 on RI or RII. Therefore, to confirm a role of PKA and to determine which PKA isozyme was responsible for the observed cAMP-dependent effects, we used the following three analogues: 8-Br-cAMP, binding preferentially to site 1 of RII; 8-AHA-cAMP, binding preferentially to site 1 of RI; and the analogous 6-Mb-cAMP, binding to site 2 in both RI and RII, and, for this reason, able to selectively activate PKA I or PKA II, depending on the analogue with which it is combined (36). Ovulated COCs were cultured in control medium or in a medium containing increasing concentrations of each of the three analogues, and apoptosis was analyzed at 7 h together with cumulus dissociation. As shown in Table 1, no inhibition or only a mild inhibition of apoptosis was obtained with 6-Mb-cAMP and 8-AHA-cAMP at any of the concentrations tested and with 8-Br-cAMP at a concentration of 50 μM. However, when 6-Mb-cAMP was combined with 50 μM 8-Br-cAMP (*i.e.* the pair of analogues putatively capable of activating PKA II), apoptosis was completely inhibited, whereas no effect was observed when 6-Mb-cAMP was administered in association with 8-AHA-cAMP (*i.e.* the couple that activates PKA I) (Table 1). As expected, the pair 8-AHA-cAMP and 8-Br-cAMP, activating R subunits of different PKA isotypes, had no effect. These results suggest that stimulation of the PKA II underlies the cAMP-promoted inhibition of cumulus cell apoptosis.

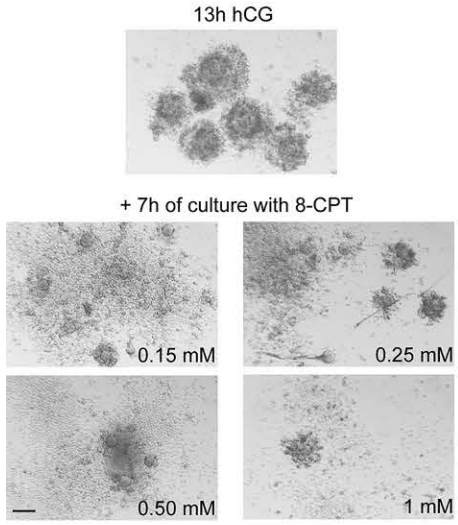

FIGURE 6. **Effects of a selective EPAC activator cAMP analogue on dissociation of ovulated COCs cultured *in vitro*.** Shown are representative micrographs of COCs collected at 13 h after hCG and cultured for 7 h i nt hepresence of different concentrations of 8-pCPT-2′-O-Me-cAMP, a selective EPAC activator. The 8-pCPT-2′-O-Me-cAMP was not able to preserve the matrix. Note that cumulus cells were shed in the medium during the culture at all concentrations tested. *Bar*, 200 μm.

TABLE 1

Effects of cAMP analogues on apoptosis and matrix dissociation in ovulated COCs cultured *in vitro*

Ovulated COCs were cultured for 7 h with 5% FCS in the absence or in the presence of increasing concentrations of each of the three analogues or with different combinations of two site-selective cAMP analogues.

Treatment	Concentration	Apoptosis[a]	DI[b]
	mM	*%*	*AU*
No culture		7 ± 2	0
None		36 ± 3.1	2
8-Br-cAMP	0.05	24 ± 4.1	2
	0.15	12 ± 7.5	0/1
	0.25	10 ± 1.5	0
	0.50	8 ± 0.9	0
	1	6 ± 1.2	0
8-AHA-cAMP	0.25	22 ± 1.5	2
	0.50	32 ± 7.8	2
	1	30 ± 3.6	2
6-Mb-cAMP	0.25	21 ± 0.5	2
	0.50	21 ± 2.5	2
	1	25 ± 4.6	2
6-Mb-cAMP + 8-Br-cAMP	0.25 + 0.05	5 ± 2	0
6-Mb-cAMP + 8-AHA-cAMP	0.25 + 0.25	30 ± 3.6	2
8-AHA-cAMP + 8-Br-cAMP	0.25 + 0.05	26 ± 1.8	2

[a] The data are the mean ± S.E. of the percentage of apoptotic CCs/COC of at least three independent experiments.
[b] DI, dissociation index of COC; based on morphological evaluation and expressed in arbitrary units (AU) from 0 (intact COC) to 4 (denuded oocyte).

Interestingly, 6-Mb-cAMP and 8-AHA-cAMP alone did not show any effect on cumulus dissociation, and, furthermore, inhibition by 8-Br-cAMP required a greater dose. In any case, only the combination of cAMP analogues activating PKA II efficiently inhibited cumulus dispersion, as found for apoptosis.

It was demonstrated previously that cAMP elevation in follicle cells activates the PI3K/Akt and MAPK signaling pathways through dependent and independent PKA mechanisms (37–39). Thus, involvement of these kinases in 8-Br-cAMP-induced cumulus cell survival was analyzed in ovulated COCs cultured *in vitro* for 7 h by usings electivei nhibitors(Fig.7 *A*). Consistent with the results reported above, H89, an inhibitor of PKA activity, completely reversed the effect of 8-Br-cAMP. Two chemically different inhibitors of PI3K, LY294002 and wortmannin, produced an acceleration of apoptosis but only partially counteracted the action of 8-Br-cAMP. Treatment of the cells with UO126, a dual inhibitor of MEK1 and MEK2, which phosphorylate and activate MAPKs, had no effect. These results suggest that PKA is the dominant kinase for long term cumulus cell survival and that it might act through multiple pathways.

Conversely, the effect of 8-Br-cAMP on matrix stability was not apparently altered by H89 or by the other inhibitors in cultured ovulated COCs (data not shown). To study this phenomenon in more detail, we performed metabolic labeling of HA during *in vitro* FSH-induced COC expansion. At 15 h of culture, inhibitors alone or in association with 8-Br-cAMP were added to the culture medium, and HA release from the matrix was analyzed 9 h later (24 h of total culture) (Fig. 7*B*). Consistent with the morphological observation, the HA retention in the matrix promoted by 8-Br-cAMP was not inhibited by either H89, LY294002, or UO126. Interestingly, treatment with H89 alone inhibited HA release as efficiently as 8-Br-cAMP. These results may reflect the ability of H89 to inhibit, in addition to PKA, also kinases that can be inhibited downstream from the PKA signaling pathway, a condition that would mimic the action of PKA (40).

8-Br-cAMP Action Does Not Require Protein Synthesis—The apoptotic pathway is regulated by the synthesis of pro- and anti-apoptotic proteins as well as by post-transcriptional modification of regulatory components and executors as caspases. We found that *in vitro* treatment of ovulated COCs for 7 h with cycloheximide, which blocks protein synthesis, enhanced apoptosis in a dose-dependent manner, inducing 80–90% cell death at the highest doses tested (Fig. 8). It is noteworthy that the 8-Br-cAMP anti-apoptotic action was highly resistant to cycloheximide, with the apoptotic rate not exceeding 20% even at the highest concentrations of the drug. Next, we examined the possibility that 8-Br-cAMP might produce a rapid and reverse modification of apoptotic regulators rather than stimulate new protein synthesis, which would occur over several hours. The time of action of 8-Br-cAMP was then assessed. First, to determine the time course of apoptosis in the absence of 8-Br-cAMP, COCs were cultured in basal conditions, and apoptosis was evaluated at different times. Results reported in Fig. 9*A* show that apoptosis rapidly increased between 3 and 5 h of culture, reaching the maximum value at 7 h. Conversely, when 8-Br-cAMP was added to the medium at 3 or 5 h of culture, no further increase of apoptosis was found at 7 h. These observations indicated that 8-Br-cAMP was able not only to inhibit the onset of apoptosis but also to promptly block this process when it was ongoing. Then we investigated whether the 8-Br-cAMP effect could be reversed. 8-Br-cAMP was added from the beginning of the culture and washed out at different times. We found that the removal of 8-Br-cAMP at any time between 2 and 24 h of culture allowed the cells to resume the death program (Fig. 9*B*).

Interestingly, dissociation of ovulated COCs was inhibited by cycloheximide, independent of the presence or the absence of 8-Br-cAMP (Fig. 8). In addition, dispersion of the cumulus was not inhibited when treatment with 8-Br-cAMP was delayed for 3 h (data not shown). These results suggest that synthesis of

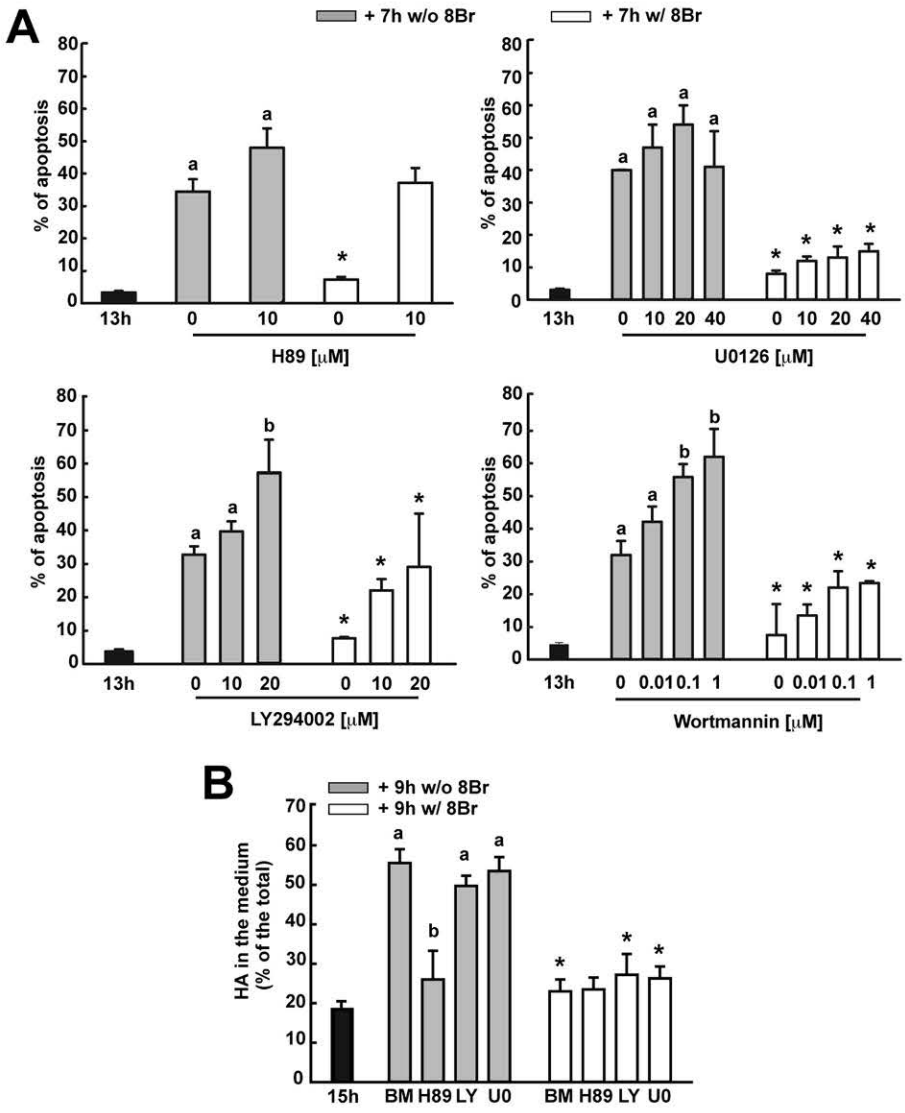

FIGURE 7. **Signaling pathways involved in the protective action of cAMP on apoptosis and matrix disassembly.** *A*, effect of PKA inhibitor H89, PI3K inhibitors LY294002 and wortmannin, and MAPKK inhibitor UO126 on apoptosis. Ovulated COCs were preincubated for 1 h with the aforementioned inhibitors and then cultured for 7 h i nt hepresence of inhibitors with or without 1 mM 8-Br-cAMP. The data represent the mean ± S.E. (*error bars*)p ercentageo fa poptotic cumulus cells/COC of at least four independent experiments. *B*, effect of H89, LY294002, and UO126 on disassembly of the HA-rich matrix synthesized during *in vitro* expansion. Compact COCs were expanded *in vitro* with 20 ng/ml FSH plus 5% FCS. At 15 h of culture, the inhibitors were added to the culture medium, and after 1 h, vehicle or 8-Br-cAMP was added, and the cells cultured for an additional 9 h. The proportion of the total HA that was released from the matrix i n the medium was assessed as described under "Experimental Procedures." The values represent the mean ± S.E. of five independent experiments. Multiple-comparison analysis (one-way analysis of variance) was performed among samples untreated and treated with different concentrations of inhibitors alone, and significant differences are indicated by *different superscript letters* ($p < 0.05$). *, significance of treatment with inhibitors and 8-Br-cAMP as compared with inhibitors alone at the same concentration.

new proteins is required for matrix remodeling and that cAMP elevation probably prevents changes that irreversibly commit their expression.

Effect of Maintenance of Cumulus Integrity on Oocyte Fertilization—Inm ammals,t hed evelopmentalc apacityo ft he oocyte is maintained only for a narrow window of time both *in vivo* and *in vitro*.I nm ice,w ithin1 2h o fo vulation,s everal molecular changes take place in the oocytes, leading to decreased fertilization rate and increased cytoplasmic fragmentation (*i.e.* default activation pathway) upon insemination (17). Because dispersion of the cumulus parallels the aging of the enclosed oocyte, we wondered whether the maintenance of cumulus integrity could prolong the fertilizability of the oocytes. To explore this question, the *in vitro* fertilization rate of ovulated COCs cultured for 14 h with and without 8-Br-cAMP was compared with that of COCs inseminated soon after ovulation (time 0 h, control). As shown in Table 2, an increase in oocyte fragmentation and decrease in two-pronucleus formation occurred in both experimental groups. However, the majority of oocytes subjected to insemination after culture without 8-Br-cAMP underwent fragmentation, and the rate of fertilization and two-cell embryo development dropped to about 30 and 25% of the control, respectively. Conversely, when COCs were cultured in the presence of 8-Br-cAMP, the proportion of normally fertilized oocytes as well as two-cell embryo formation was improved, reaching about 70 and 50% of control, respectively. The possibility that 8-Br-cAMP exerted a direct effect on oocytes is unlikely because this cyclic nucleotide did

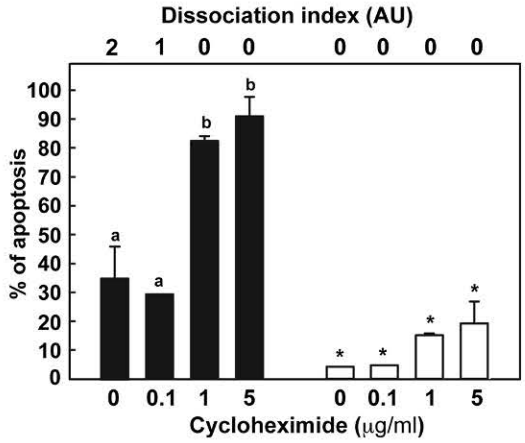

FIGURE 8. **Prevention of spontaneous apoptosis by 8-Br-cAMP is protein synthesis-independent.** Ovulated COCs were preincubated for 1 h with different concentrations of cycloheximide, an inhibitor of protein synthesis, and cultured for 7 h i nt hepresenceo ft hei nhibitorw ithout(*solidb ars*)o rw ith (*empty bars*) 1 mM 8-Br-cAMP. The data represent the mean ± S.E. (*errorb ars*) percentage of apoptotic cumulus cells/COC of at least four independent experiments. Multiple comparison analysis (one-way analysis of variance) was performed among samples untreated and treated with different concentrations of cycloheximide alone, and significant differences are indicated by *different superscript letters* ($p < 0.05$). *, significance of treatment with the cycloheximide and 8-Br-cAMP as compared with cycloheximide alone at the same concentration. The dissociation index of the COC, reported at the *top*, was based on the morphological evaluation of cumulus cell shedding and expressed in arbitrary units (*AU*) from 0 (intact COC) to 4 (denuded oocyte).

not ameliorate the fertilization rate of denuded oocytes cultured *in vitro*.

Discussion

Data reported in this paper show that cumulus cell apoptosis dramatically increases in the oviduct a few hours after ovulation, paralleling matrix remodeling. Both processes were inhibited *in vitro* by the treatment with either cAMP analogues or by elevating endogenous intracellular cAMP with adenylate cyclase activator forskolin or phosphodiesterase inhibitor rolipram. Notably, the maintenance of COC integrity by cAMP prolonged the fertile life span of ovulated oocytes. These findings provide the first evidence that modulation of cAMP in cumulus cells dictate the time of their survival and consequently the time frame of oocyte fertilization.

Several lines of evidence indicate that, in addition to triggering luteinization of granulosa cells, LH promotes granulosa cell survival during the time preceding ovulation. The observation that cAMP analogues, such as LH, prevent the spontaneous onset of granulosa cell apoptosis in cultured preovulatory follicles suggests that this hormone exerts its protective role mainly via a cAMP-dependent signaling pathway (29). Data reported in this paper suggest that a cAMP-dependent protective mechanism also operates in cumulus cells during the periovulatory follicles. FSH and EGF are able to induce, in concert with oocyte factors, *in vitro* cumulus expansion, which signals the final differentiation of cumulus cells. These compounds elicit different membrane transduction signals, tyrosine kinase-dependent for EGF and cAMP-dependent for FSH, which probably both converge in activation of MAPKs for stimulating such a process (41, 42). In addition, the stimulation of cumulus cells with either FSH and EGF during *in vitro* oocyte maturation enhances

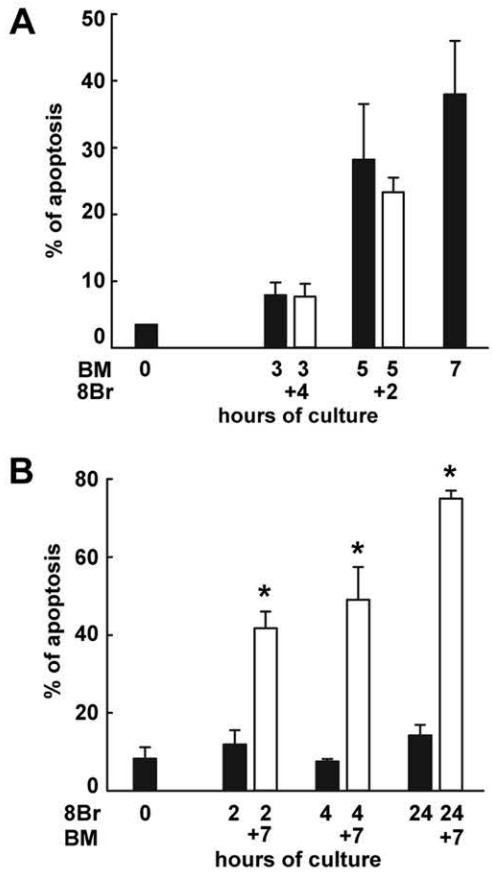

FIGURE 9. **The cAMP agonist 8-Br-cAMP exerts a rapid and reversible action on cumulus cell apoptosis.** *A*, ovulated COCs were cultured *in vitro* with 1 mg/ml BSA (basal medium (*BM*)), and apoptosis was evaluated at 0, 3, 5, and 7 h of culture(*solid bars*). In another series of cultures, 8-Br-cAMP was added to the medium at 3 or 5 h, and COCs were cultured for an additional 4 and 2 h, respectively (*i.e.* for a total of 7 h o fculture),b eforea poptosise valuation (*empty bars*).N otet hatt hea poptosisr atea tt otal7 -hc ulture(3 + 4 or 5 + 2) was not significantly different from that at the time of the 8-Br-cAMP addition (3 or 5 h) ($p = 0.09$). *B*, ovulated COCs were cultured in the presence of 1 mM 8-Br-cAMP, and apoptosis was evaluated at 2, 4, and 24 h of culture (*solid bars*). In another series of cultures, 8-Br-cAMP was washed out at 2, 4, and 24 h, and COCs were cultured for an additional 7 h before apoptosis evaluation (*empty bars*). Apoptosis was significantly increased 7 h after 8-Br-cAMP removal at each time culture (*, $p < 0.01$). *Error bars*, S.E.

oocyte developmental competence, as evidenced by the increased rate of embryo and fetal development (43–47). The molecular mechanisms underlying the beneficial effect of these two compounds on oocyte began to be elucidated. It has been recently shown that EGF and EGF-like growth factors are able to generate signals by cumulus cells that control in oocytes the translation of mRNAs relevant for meiotic progression and development (5). We now show that FSH sustains cumulus cell survival. In fact, COCs treated *in vitro* with EGF show a significantly higher incidence of apoptotic cumulus cells than COCs expanded *in vivo*,w hereasc AMPa naloguesa sw ella sF SH mimicked the *in vivo* condition sustaining the survival of cumulus cells throughout the process of cumulus maturation. It should be pointed out that FSH was required to sustain cumulus cell survival although serum was present, a condition that is routinely used for *in vitro* maturation of mammalian COCs, including human COCs. These results strongly support the benefit of combining EGF and FSH during COC maturation. It is likely that FSH exerts an important role *in vivo*, a s*inv itro*, i n

TABLE 2

Comparison of fertilization and development of cumulus-enclosed and denuded oocytes cultured for 14 h with and without 8-Br-cAMP

Percentage of fragmented oocytes and fertilized oocytes evaluated at 6 h from insemination and percentage of two-cell embryos of the total inseminated oocytes evaluated at 24 h. COC, cumulus cell-oocyte complex (cumulus-enclosed); DO, oocytes denuded of cumulus cells; FR, fragmented oocytes; 2PN, fertilized oocytes (two pronuclei); 2CE, two-cell embryos. *a*, values represent the mean ± S.E. of five independent experiments. Statistical analysis of experiments was performed with one-way analysis of variance. Differences at $p < 0.05$ were considered significant. *b versus c*, $p < 0.001$; *b versus d*, $p > 0.05$; *c versus d*, $p < 0.01$; *e versus f*, $p < 0.001$; *e versus g*, $p < 0.01$; *f versus g*, $p < 0.05$.

	Culture time	Treatment	Inseminated oocytes	FR	2PN	2CE
	h			%	%	%
COC	0		383	6.7 ± 3.1^a	47.2 ± 2.6^b	44.6 ± 4.8^e
	14	None	397	25.5 ± 7.1	14.8 ± 3.7^c	11.1 ± 3.2^f
	14	8-Br-cAMP	515	22.7 ± 0.6	33.8 ± 4.1^d	23.9 ± 1.6^g
DO	0		187	5.3 ± 3.3	27.8 ± 1.8	25.9 ± 7.9
	14	None	205	15.6 ± 6.7	6.1 ± 1.6	2.6 ± 1.4
	14	8-Br-cAMP	216	14.6 ± 3.8	5.8 ± 1.0	2.7 ± 2.6

protecting cumulus cells during spontaneous cycles. In fact, a simultaneous surge of circulating FSH is associated with that of LH before ovulation, and cumulus cells, conversely to granulosa cells, almost exclusively express FSH receptors. It is noteworthy that the apoptotic rate of cumulus cells in humans has been associated with poor embryo implantation and pregnancy outcome and proposed as a negative marker for selecting oocytes (48).

By the time of ovulation, matrix deposition ceases, and COCs are transferred to the oviduct, where, shortly after, cumulus matrix disorganization occurs (24). We show here that this matrix changes are associated with progressive cumulus cell death. Seven hours after ovulation, about 30% of the cells showed advanced signs of degeneration, and extracellular matrix had lost most of its elasticity. These events seem to depend on an intrinsic program activated at the final stage of cumulus cell differentiation rather than by adverse oviductal environment. This conclusion is supported by the evidence that freshly ovulated COCs cultured *in vitro* undergo apoptosis and matrix disassembly with the same temporal course and at the same rate as COCs maintained in the oviduct. In addition, COCs stimulated *in vitro* with FSH undergo the same changes after completion of expansion, albeit at a lower rate. In this culture condition, almost half of the HA accumulated at the end of cumulus expansion was released from the matrix to the medium during the following 9 h o fculture,a nda bout2 0%o f cells showed apoptotic features.

A decrease of the intracellular cAMP level appears to be functional in the execution of this program. This hypothesis is supported by the evidence that, when 8-Br-cAMP was added to FSH-expanded COCs, no significant HA release or apoptosis occurred within the following 9 h o fculture,a ndm osto ft he cells were still healthy and associated with the oocyte by an elastic matrix even at 24 h after expansion completion. A similar effect was obtained by culturing ovulated COCs in the presence of forskolin, but not in the presence of FSH, indicating that, although ovulated cumulus cells have adequate adenylate cyclase machinery, the hormone failed to activate it. This can be explained by a decreased responsiveness of expanded cumulus cells to this hormone. In fact, it is well known that stimulation of follicle cells with an ovulatory dose of gonadotropins leads to adenylate cyclase desensitization for several hours to further hormone stimulus. In agreement, we have shown previously that FSH stimulates maximum cAMP production by COCs within 2 h o fculture,w hereasa p rogressived eclineo ccurs afterward toward the basal level, although FSH was continuously present (49). Finally, a comparative study of gene expression profiles at different stages of cumulus maturation has shown that expression of FSH receptor is down-regulated in COCs after ovulation (50).

The intracellular pathway by which elevation of cAMP prevents matrix disassembly and apoptosis in the cumulus was investigated by site-selective affinity cAMP analogues (35, 36). The ineffectiveness of 8-pCPT-2′-*O*-Me-cAMP, which selectively binds and activates EPAC, suggests that this intracellular pathway is not involved in controlling cumulus integrity. Only a paired combination of cAMP analogues that synergistically activate the type II PKA was able to recapitulate the effect of the adenylate cyclase activator forskolin, whereas selective PKA I agonists had no effect. This result is in agreement with previous findings suggesting a compartmentalization of the PKA isozymes in the COC with type I PKA being expressed in the oocyte and type II PKA being present in the cumulus cells (51). Type II regulatory subunits show high affinity for several anchoring proteins (AKAPs), which determine different enzyme intracellular localization and action specificity (52). Interestingly, apoptosis and matrix disassembly showed different sensitivity to intracellular cAMP level, because the 8-Br-cAMP minimum effective dose was lower for the former than for the latter, suggesting that these processes are probably controlled by differently sequestered PKA II.

The relevance of PKA activity in preventing cumulus cell apoptosis was confirmed by the ability of the inhibitor H89 to completely reverse the protective effect of 8-Br-cAMP. It has been reported previously that cAMP/PKA can affect the PI3K/AKT and MAPK signaling pathways (37–39), which promote cell survival under several proapoptotic stimuli. However, the failure of the inhibitor of MEK1/MEK2-dependent MAPKs UO126 to influence the apoptosis both in the absence and in the presence of 8-Br-cAMP makes the participation of ERK1 and -2 in the control of this process unlikely. Noteworthy, inhibition of PI3K with both LY294002 and wortmannin accelerated the apoptosis of cumulus cells in control conditions but only partially inhibited the protective action of 8-Br-cAMP, suggesting that although the PIK3 activity is important for cumulus cell survival, PKA acts through a PI3K-dependent and independent pathways. Previous studies have shown that PKA modulates apoptosis either by modulating the expression of antiapoptotic

or proapoptotic effectors or directly targeting the phosphorylation of apoptotic modulators or effectors at the cytoplasmic and at the mitochondrial level, leading to their sequestration or ubiquitination (53–55). It is likely that a post-transcriptional action of PKA is operating in the process of cumulus cell apoptosis because the protective effect of 8-Br-cAMP was protein synthesis-independent, being resistant to cycloheximide treatment. In agreement, the administration of 8-Br-cAMP to the COC cultures when apoptosis was ongoing completely blocked further apoptotic progression, and its removal at any time of culture induced apoptosis, indicating that the action of 8-Br-AMP was rapid and reversible.

The action of 8-Br-cAMP on matrix dissociation differs from that on apoptosis in several aspects. Conversely to apoptosis, matrix dissociation was prevented by cycloheximide, and early, but not late, administration of 8-Br-cAMP to the cultures was effective (data not shown). Thus, new proteins are required for matrix remodeling, and the elevation of cAMP probably prevents their expression. Furthermore, 8-Br-cAMP-dependent inhibition of HA release from the matrix was not counteracted by any of the kinase inhibitors tested. Moreover, the ineffectiveness of H89 was in contrast to the overall results reported above, calling into question the involvement of PKA. However, the observation that treatment with H89 alone inhibits HA release as efficiently as 8-Br-cAMP might explain this contradictory result, suggesting an involvement of the RhoA/ROCK signaling pathway in PKA regulation of this process. Actually, it has been demonstrated that the inhibitor of PKA H89 also diminishes the activity of ROCK II with similar IC_{50} when tested in a cell-free assay (40, 56) and that, in the cells, PKA can indirectly inhibit ROCK by phosphorylating and inactivating its stimulator RhoA (56, 57). Thus, the evidence that H89 mimics rather than inhibits cAMP (PKA)-dependent matrix-stabilizing activity suggests that ROCK inhibition is a downstream effect of PKA activity in cumulus cells. Rho/ROCK activates myosin II, thereby inducing the rearrangement of cortical actin microfilaments, which results in retraction of cytoplasmic projections and changes in cell membrane-extracellular matrix interaction. PKA has the opposite effect on the cytoskeleton, thereby promoting the formation and elongation of cytoplasmic projections in several cell types (57–59). In agreement with the advanced hypothesis, an extensive network of cellular projections is formed by cumulus cells under the gonadotropin stimulus, and disruption of actin cytoskeleton prevents the formation of the expanded matrix (60). In addition, the necessity to restrain RhoA activation to achieve full cumulus expansion has recently been proven by the evidence that inhibition of prostaglandin E_2/cAMP signaling in prostaglandin E receptor EP2-deficient mice increases RhoA activation in cumulus cells and leads to alteration of cell shape and matrix organization (61). These findings, together with results reported here, suggest that cAMP-dependent inhibition of the RhoA/ROCK/myosin-actin pathway is relevant for correct assembly of the matrix and for retention of HA at the cumulus cell surface. Whatever the mechanism, the finding that apoptosis occurs in the presence of H89 although HA release is inhibited further suggests that the two processes are triggered at the same time but through distinct pathways, which are both inhibited by PKA.

However, the onset and progression of apoptosis coincide well with cumulus matrix dissociation, suggesting that deprivation of anchorage might contribute to ensure cumulus cell death. This hypothesis is also supported by the evidence that healthy cells released during cumulus disaggregation survive for days if they adhere to the plastic, thereby reverting their commitment to apoptosis (23). The observation that no significant degradation of HA occurs during cumulus dispersion (23) suggests that proteins involved in HA organization become degraded. Pericellular proteolytic degradation of adhesive proteins and consequent integrin-cytoskeleton modifications have been implicated in facilitating apoptosis in pathological conditions (62) and in physiological processes, such as involution of mammary glands after lactation and remodeling of tissues during morphogenesis (63–65). Interestingly, adhesive molecules have been detected in proximity to cumulus cell surface in ovulated COCs (50, 60, 66), and they can indirectly contribute to anchorage HA to the cell membrane by binding to the G3 domain of versican, an HA-binding proteoglycan present in the cumulus matrix (67, 68). Proteases implicated in the cleavage of adhesive proteins include tissue plasminogen activator, urokinase plamsinogen activator, matrix metalloproteinases, membrane-type metalloproteinases, and a disintegrin and metalloproteinase with thrombospondin motifs (69). Notably, mouse cumulus cells dramatically increase the expression and enzymatic activity of urokinase plamsinogen activator after completion of cumulus expansion both *in vivo* and *in vitro*,s uggesting an active role of this protease in postovulatory dissolution of the cumulus (24). In addition, postovulatory oocytes release significant amounts of the proteolytic enzyme tissue plasminogen activator, which can contribute to destabilization of the cumulus matrix (24). However, these enzymes are not the sole proteases involved in the cumulus matrix degradation, and they may have a redundant role. In fact, we have found that matrix disorganization and cumulus apoptosis also occur when *in vivo* and *in vitro* expanded cumuli were cultured in the absence of oocytes or in the absence of serum, which is the source of plasminogen. ADAMTS-dependent versican proteolysis has been linked to apoptosis during limb bud development (64), and versican processing also occurs during the last phase of cumulus expansion by the increased expression of ADAMTS1 (70, 71). Certainly, versican cleavage can help loosen the HA strand network, thereby altering the interaction of HA with adhesive proteins and with cell surface receptors. Indeed, a growing body of evidence suggest that HA can also directly participate in sustaining the vitality of several cell types by binding to HA cell surface receptors, mainly to CD44 (72–75). This molecular interaction seems also to take part in the control of cumulus survival. Cumulus cells stimulated by gonadotropin to express HA also express CD44 in all examined mammalian species, including humans (50, 76–78). In addition, exogenous HA decreases the incidence of apoptosis in human cumulus cells pretreated with hyaluronidase (*i.e.* after removal of endogenous HA) and cultured *in vitro* (79), and inhibition of HA-CD44 binding with anti-CD44 antibody prevents this effect and leads to decrease of procaspase-3, -6, and -9 levels in porcine cumulus cells, suggesting that perturbation of HA-CD44 interaction leads to the activation of these caspases (80). Accordingly, the

cytoplasmic tail of CD44 interacts with actin-cytoskeleton adaptor proteins (81, 82), and rearrangement of actin filaments is involved in the cell death following loss of cell anchorage by releasing apoptotic activators (*i.e.* Bim and Bmf), sequestered by cytoskeleton-bound proteins (83, 84). Intracellular signals generated by CD44 change in function of HA concentration (85, 86). In this regard, it should be pointed out that HA is produced at the plasma membrane by HA synthases. Therefore, HA polymers elongating by HA synthases at the cell surface can significantly contribute to the stiffness of this molecule in proximity to the cell membrane. Accordingly, we found that cumulus cell apoptosis occurs shortly after HA synthesis ceases and concomitantly with the beginning of HA release from the matrix. Thus, it is reasonable to hypothesize that decreased synthesis and increased disorganization of HA in proximity to the cells at the end of cumulus expansion play a role in the induction of cumulus cell apoptosis. Further studies are required to clarify how the intra- and extracellular pathways are integrated to cause cumulus cell apoptosis at the appropriate time after ovulation.

The cumulus oophorus is essential for *in vivo* oocyte fertilization. Deletion of genes involved in cumulus matrix synthesis and organization results in severe subfertility or complete sterility of female mice (7). Indeed, cumulus cells deeply influence the behavior of both oocyte and sperm in the oviduct. Glycolysis is defective in oocyte even after fertilization, and cumulus cells nurse the germ cells and the early stage embryos by providing them with pyruvate (87). In addition, soluble products as well as matrix components secreted by cumulus cells stimulate the motility, promote the capacitation, and initiate the acrosome reaction in the crossing spermatozoa, making them suitable for fertilization (15, 16). In agreement with these observations, it has long been demonstrated that a decrease of the oocyte fertilization rate occurs in parallel with a reduction of cumulus cell mass at a later time after ovulation (22). Results reported here further support the important role of cumulus cells in sustaining fertilization. We found that cAMP elevation prolongs the integrity of the cumulus, preventing cumulus cell apoptosis and matrix disassembly. More important, we demonstrated that cAMP-stabilized cumuli retain their functional properties, prolonging the fertilizability of oocytes retrieved after ovulation and cultured *in vitro*.O vernightc ultureo fo vulated COCs produced a 70% reduction in normally fertilized oocytes, but when 8-Br-cAMP was added to the culture, the decrease was only 30%, giving rise to almost double the number of two-cell embryos.

In conclusion, we have demonstrated that cAMP-elevating agents prevent disaggregation of the cumulus oophorus and sustain the survival of cumulus cells, allowing them to continue to exert beneficial effects on oocyte and sperm, thereby extending *in vitro* the time frame of oocyte fertilizability. Cumulus cells exert a positive effect on oocyte maturation also in humans (88–91), and human cumulus cell apoptosis has been correlated with a low potential development of the enclosed oocyte (48). Therefore, these results might be of relevance in clinical practice to optimize the conditions for promoting and preserving the quality of oocytes during handling and culture of COCs retrieved from patients enrolled in programs of assisted reproduction. In addition, it will be of interest to determine whether the dual action of cAMP in the cumulus is exerted also on other HA-rich tissues, particularly at the inflammation site, where hyaluronan is expressed (92) and the protective effect of cAMP elevation through phosphodiesterase inhibitors has been proven (93–95).

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Cyclic AMP elevating agents promote cumulus cell survival and hyaluronan-matrix stability thereby prolonging the time of mouse oocyte fertilizability

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Supplemental Data

Movie S1. COCs were kept in a drop of medium and the movies captured during repeated aspiration and release of the culture medium. Observation of the elasticity of the cumulus matrix at 15 h of culture with FSH. Note that the cumulus mass becomes flattened under compression and resumes its original three-dimensional shape after the release of the applied force.

Movie S2. At 40 h of culture with FSH, the elastic matrix was completely disassembled and the cumulus cells dissociated from the oocyte. Oocytes freely floated in the medium and cumulus cells were densely packed and began spreading on the plastic.

Movie S3. The movie shows the elasticity of the matrix of COCs cultured with 8Br-cAMP for 15 h.

Movie S4. At 40 h of culture with 8Br-cAMP, the elastic matrix was preserved and the embedded cumulus cells still surrounded the oocyte.

Cyclic AMP-elevating Agents Promote Cumulus Cell Survival and Hyaluronan Matrix Stability, Thereby Prolonging the Time of Mouse Oocyte Fertilizability

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