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Rodent oocytes express an active adenylyl cyclase required for meiotic arrest

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Abstract

The intracellular levels of cAMP play a critical role in the meiotic arrest of mammalian oocytes. However, it is debated whether this second messenger is produced endogenously by the oocytes or is maintained at levels inhibitory to meiotic resumption via diffusion from somatic cells. Here, we demonstrate that adenylyl cyclase genes and corresponding proteins are expressed in rodent oocytes. The mRNA coding for the AC3 isoform of adenylyl cyclase was detected in rat and mouse oocytes by RT-PCR and by in situ hybridization. The expression of AC3 protein was confirmed by immunocytochemistry and immunofluorescence analysis in oocytes in situ. Cyclic AMP accumulation in denuded oocytes was increased by incubation with forskolin, and this stimulation was abolished by increasing intraoocyte Ca^{2+} with the ionophore A23187. The Ca^{2+} effects were reversed by an inhibitor of Ca^{2+}, calmodulin-dependent kinase II. These regulations of cAMP levels indicate that the major cyclase that produces cAMP in the rat oocyte has properties identical to those of recombinant or endogenous AC3 expressed in somatic cells. Furthermore, mouse oocytes deficient in AC3 show signs of a defect in meiotic arrest in vivo and accelerated spontaneous maturation in vitro. Collectively, these data provide evidence that an adenylyl cyclase is functional in rodent oocytes and that its activity is involved in the control of oocyte meiotic arrest.

Keywords: cAMP; Adenylyl cyclase; Oocyte; Meiosis; Cell cycle

Introduction

One of the major unsolved issues in reproductive biology is the understanding of the signals involved in the control of oocyte meiotic arrest and maturation (Conti et al., 2002). In the growing follicle, mammalian oocytes are arrested in the dictyate stage of meiotic prophase, a state that resembles a G_2/M transition. In the nucleus of these oocytes (germinal vesicle, GV), chromatin is partially decondensed and surrounds a prominent nucleolus (Tsafriri and Dekel, 1994; Wassarman and Albertini, 1994). In addition, the diffuse microtubule network in the cytoplasm resembles that of a cell in interphase (Albertini, 1992). Resumption of meiosis occurs only after the gonadotropin LH surge and involves the dissolution of the nuclear membrane (GVBD), chromosome condensation followed by orientation in the equatorial plate, and organization of the microtubules in a spindle. The reentry into the cell cycle culminates with the extrusion of the first polar body and is immediately followed by the second meiotic division in the absence of an S-phase and DNA duplication and an arrest at the metaphase II stage (Tsafriri and Dekel, 1994).

The oocyte meiotic arrest is established during fetal development and is thought to be initially dependent on factors intrinsic to the oocyte (Sorensen and Wassarman, 1976). The oocyte is incompetent to undergo maturation because key components of the cell cycle machinery are maintained below a threshold or are completely absent (Chesnel and Eppig, 1995; Mitra and Schultz, 1996). Competence to resume meiosis is acquired during oocyte growth well before the LH surge, at a stage of follicular development that roughly corresponds to, or immediately precedes,

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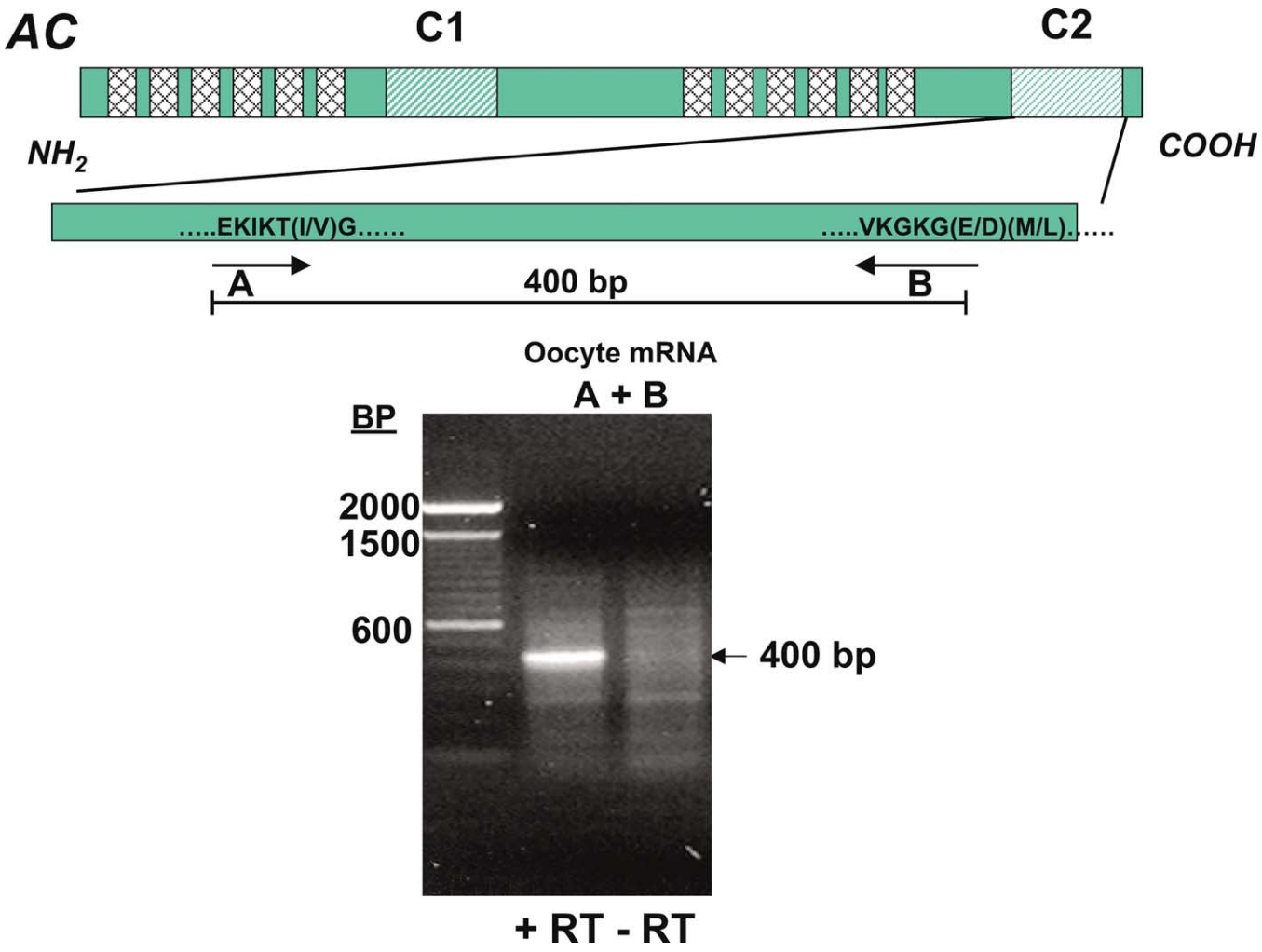


Fig. 1. RT-PCR analysis of AC mRNA expressed in rat oocyte. A schematic representation of the strategy used to amplify AC transcripts from oocytes is reported at the top of the figure. The mRNA from denuded rat oocytes was reverse transcribed into cDNA and used as a template for PCR amplification with degenerate primers A and B. The products of the amplification are shown in the lower panel, along with a negative control with no reverse transcriptase. PCR products were analyzed on a 1.2% agarose gel stained with ethidium bromide.

the appearance of the antrum (Sorensen and Wassarman, 1976). Since oocytes remain quiescent in preovulatory follicles, the hypothesis has been put forward that the meiotic arrest of competent oocytes is dependent on inhibitory constraints present in the follicular environment (Eppig, 1993). Several candidate molecules produced by cumulus or granulosa cells have been proposed to fulfill this role of meiotic inhibitor, including the purine metabolite hypoxanthine (Downs, 1997) or a putative factor termed OMI (Tsafriri and Pomerantz, 1986).

Although the nature of the signal arising from granulosa cells necessary to maintain the meiotic arrest is still debated, it is widely accepted that the concentration of the second messenger cAMP within the oocyte plays a critical role in maintaining this state (Conti et al., 2002). In mammalian and amphibian oocytes, an array of experimental paradigms has shown that high cAMP steady state levels prevent spontaneous resumption of meiosis (Cho et al., 1974; Maller and Krebs, 1980; Pincus and Enzmann, 1935). This view is reinforced by the finding that resumption of meiosis is associated with a decrease in cAMP (Dekel et al., 1984; Schultz et al., 1983; Vivarelli et al., 1983).

Over the years, a major point of contention has been the source of the cAMP that accumulates in the oocytes (Tsafriri and Dekel, 1994). It has been proposed that oocytes do not produce cAMP and the cyclic nucleotide is provided by adjacent cumulus cells via diffusion through gap junctions (Gilula et al., 1978; Tsafriri and Dekel, 1994). According to this view, cAMP itself may be the meiotic inhibitor provided by granulosa cells, and a decrease in oocyte cAMP may be caused by closure of the gap junctions between somatic cells and oocytes. Alternatively, the oocyte itself produces sufficient cAMP to maintain a meiotic arrest, and that extrinsic factors stimulate the synthesis of this second messenger in the oocytes (Eppig, 1993). As a corollary to this second scenario where granulosa cells actively participate in the meiotic arrest, the LH surge serves to relieve this blockade. Consistent with the latter postulate is the recent

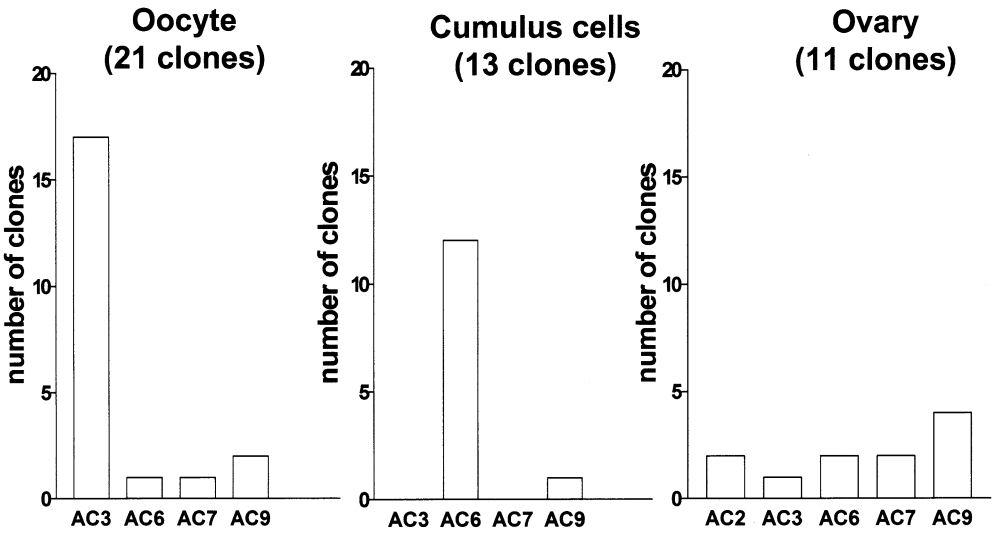


Fig. 2. Adenylyl cyclase isoforms detected in the rat ovary. RT-PCR products amplified by degenerate primers for all nine forms of adenylyl cyclases with cDNA derived from rat ovary and isolated oocytes and cumulus cells were cloned into TOPO vector for sequencing. AC3 was the major form of adenylyl cyclase found in oocytes, while the majority of AC clones in cumulus cells were AC6. The clones were obtained from three different PCRs, each with different RNA and cDNA preparations.

observation that inhibition of Gs in situ with inhibitory antibodies causes meiotic resumption in the intact follicle (Mehlmann et al., 2002). Several additional studies have addressed this issue by using forskolin to stimulate adenylyl cyclase, but results have been inconsistent (Dekel et al., 1984; Ekholm et al., 1984; Racowsky, 1984). The presence of adenylyl cyclase activity in bovine cumulus–oocyte complexes has been suggested by cytochemistry (Kuyt et al., 1988). Some of these findings have been regarded with skepticism because of the difficulty in isolating oocytes free of remnants of cumulus cell plasma membranes.

The recent observation that PDE3A is required for meiotic resumption and that an increase in PDE activity is observed during GVBD argues in favor of the presence of an active mechanism regulating meiotic maturation (Richard et al., 2001; Tsafriri et al., 1996). In the course of these studies, it was observed that incubation of denuded oocytes with PDE3 inhibitors causes a substantial increase in cAMP, suggesting that the oocyte is able to synthesize cAMP on its own. Here, we have reexamined the issue by investigating the expression of adenylyl cyclase genes and their function in the oocyte. Using different approaches, we provide evidence that rat and mouse oocytes express AC3 and that this enzyme contributes to the control of cAMP levels in the gamete.

Materials and methods

Oocyte collection

Immature (24- to 25-day-old) Sprague–Dawley rats (Simonsen Laboratories, Gilroy, CA) were injected s.c. with 10 I.U. PMSG (Calbiochem, San Diego CA), or C57/BL6 × 129 mice were injected i.p. with 5 I.U. PMSG. After 45–48 h, ovaries were excised and collected in warm (37°C) Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/ml penicillin and streptomycin. Cumulus cell-enclosed oocytes were collected from punctured follicles, separated from granulosa cells by serial dilutions in medium, then stripped of cumulus cells by repeated pipetting with a small-bore pipet. The largest denuded oocytes were cleaned of all granulosa cells by successive washings with complete medium, then collected in 3 μl, to be used either for RNA isolation or for culture.

RT-PCR

Denuded oocytes were washed in phosphate-buffered saline (PBS), diluted with 3× volume of Trizol reagent (Invitrogen) and stored at −80°C. For RNA isolation, 1000 oocytes were pooled and processed according to the protocol for Trizol reagent. RNA from granulosa cells, cumulus cells, and entire ovaries was also isolated by using Trizol reagent. A comparison of RT-PCR with RNA treated with DNase showed no contamination of DNA in the RNA preparations. RT reactions were performed with 1-5 μg RNA and random hexamers. For use in PCR, the resulting cDNA from equal amounts of RNA for each preparation was precipitated with ethanol. To confirm the presence of adenylyl cyclases in different compartments of the ovary, degenerate primers (5′-G/AA/CG/C/AAAG/AATC/TAAG/AACCA/GTT/C/AGG-3′ and 5′-ATC/TT/CC/TC/ACCT/CTTNCCC/TTTC/GAC-3′), which amplify all forms of rat adenylyl cyclases, were used. The PCR conditions (50 cycles of 94°C for 4 s, 37°C for 1 min, 55°C for 3 min, then elongation at 72°C for 10 min) were as previously described (Gautier-Courteille et al., 1998). A band of approximately 400 bp was purified from a 1.5% agarose gel

and subcloned into TOPO TA vector (Invitrogen). The nucleotide sequences (performed by Biotech Core, Mountain View, CA, with an ABI automated DNA sequencing facility) of clones from the various cell types identified the isoforms of adenylyl cyclase expressed. In addition, a PCR with specific primers for AC3 (5′-AGATTCATGGACCCAGAGATGGA-3′ and 5′-CGGGATCCTTGTGGTCGTATTCA TCAAA-3′) (Xia et al., 1992) was also performed for 5 min at 94° C, then 5 cycles of 94° C for 1 min, 52° C for 2 min, and 72° C for 2 min, then 40 cycles of 94° C for 1 min, 55° C for 2 min, and 72° C for 2 min, then 72° C for 7 min. Bands of 570 bp were purified from agarose gels, cloned into TOPO TA vectors, and sequenced.

In vitro oocyte maturation studies

Denuded oocytes were obtained as described above and maintained in 1 ml of Leibovitz's L-15 medium supplemented with 5% heat-inactivated fetal bovine serum and 100 μg/ml penicillin and streptomycin in a double-welled culture dish with the outer well filled with water for humidity control. Incubations were carried out at 37° C for up to 24 h in a Billups–Rothenberg incubation chamber. The progression of meiotic maturation was scored by observing the breakdown of germinal vesicles (GVBD) and the subsequent appearance of polar bodies with an inverted microscope fitted with a Hoffman contrast lens.

Cyclic AMP measurements in oocytes

Denuded rat oocytes were collected and incubated at 37°C for 30 min in complete medium with the addition of 1 or 10 μM cilostamide (Calbiochem), and in some cases 50 μM forskolin (Sigma, St. Louis, MO), 10 μM of the calcium ionophore A23187(Sigma), 4 mM $CaCl_2$, and/or 10 μM KN-62 (Calbiochem), a Ca^{2+}, calmodulin-dependent kinase (CaM kinase II) inhibitor. The oocytes were collected in 3 μl of medium, which was then frozen and thawed to break the cells, and 100 μl of 0.1% TCA in 95% ethanol was added. Samples were then centrifuged at 3000 rpm for 10 min at 4°C, and the supernatant was evaporated while centrifuging under vacuum, and reconstituted with 100 μl of PBS. Cyclic AMP was measured by the radioimmunoassay method of Harper and Brooker (1975).

Analysis of the phenotype of the AC3-deficient mice

$AC3^{+/-}$ mice provided by Daniel Storm from the University of Washington Animal Facility were used to generate $AC3^{-/-}$ mice to study the maturation of oocytes devoid of AC3 (Wong et al., 2000). To genotype the mice, PCR was performed on genomic DNA extracted from tails of the offspring. The primers 5′-cctgtgctctagtagcttacgg-3′ and 5′-ctgtgaagtaggttcctacctg-3′ identified the mutant allele and 5′-ctggtgaagtggcttgacct-3′ and 5′-gttatgaagaaggagaagaca-3′ corresponded to the wild type allele.

Histology and immunohistochemistry of rat and mouse ovaries

Ovaries of PMSG-stimulated mice or rats were fixed in Bouin's solution for 4 h at room temperature, then dehydrated and stained with eosin. After embedding in Paraplast (Sigma), 6 μm sections were cut, deparaffinized, rehydrated, and then stained with hematoxylin and eosin. Slides were cleared with xylene, mounted, and analyzed by light microscopy with a Zeiss microscope fitted with AxioCam.

AC3 protein expression was visualized by immunocytochemical detection with the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Ovaries were fixed in Bouin's solution, embedded in paraffin, and then cut into 5-μm sections. Deparaffinized sections were rehydrated and rinsed in PBS, and endogenous peroxidases were blocked by incubation in hydrogen peroxide, followed by incubation in normal horse serum. The sections were then incubated overnight in an anti-AC3 antibody (1:80; Santa Cruz Biotechnology, Santa Cruz, CA) in a humidified chamber at 4°C. The distribution of the primary antibody was revealed with a biotinylated horse anti-rabbit secondary antibody, and the avidin–biotin–peroxidase complex was visualized with DAB. Sections were rinsed in PBS between each step. Slides were counterstained with hematoxylin prior to dehydration. For some sections, AC3 was localized by immunofluorescence with a Texas Red horse anti-rabbit secondary antibody, and the nuclei were stained with DAPI added to the mounting medium (Vector Laboratories). The specificity of the AC3 staining was checked by replacing the antibody with nonimmune IgG and by blocking the binding of the primary antibody with an excess of peptide (Santa Cruz Biotechnology). Endothelial cells from vessels in the same sections were used as positive controls.

In situ hybridization

Two partial segments, 387 bp (3301-3687 bp) and 421 bp (3705-3285 bp), of rat AC3 cDNA (M55075) subcloned in pcDNA3.1/V5-His-TOPO vector were used as templates for the synthesis of radioactive ^{35}S-labeled and digoxigenin (DIG)-labeled RNA probes. The plasmids containing the 387-bp and the 421-bp fragments were linearized by *Bam*HI and *Eco*RV (Roche, Indianapolis, IN), respectively. T7 RNA polymerase (Promega, Madison, WI) was used to synthesize both antisense and sense RNA probes. DIG-labeled probes were synthesized with T7 RNA polymerase in the presence of DIG RNA labeling mixture (Roche Diagnostics) according to the procedures recommended by Roche Diagnostics.

Ovaries from PMSG-primed rats were fixed in 4% paraformaldehyde (PFA) for 6 h and incubated in 0.5 M sucrose overnight at 4°C. The ovaries were embedded in OCT (Tissue-Tek, Torrance, CA), cut into 10 μm sections, and mounted. Slides were postfixed in PFA and treated in the following solutions: 0.2 M HCl, 2× SSC at 70°C, pronase E (Sigma), 2 mg/ml glycine, and triethanolamine (0.1 M,

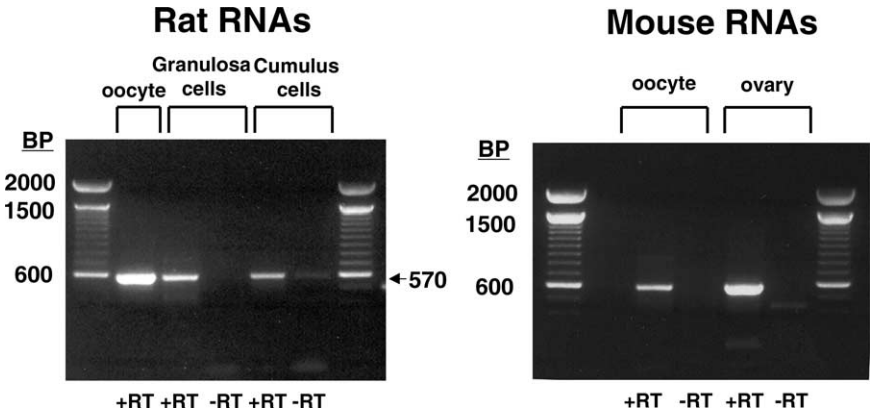


Fig. 3. Detection of AC3 mRNA in somatic and germ cells of the ovary using specific primers. RT-PCR of mouse and rat RNA from ovary, oocytes, cumulus cells, and granulosa cells was performed by using primers specific for AC3. The expected 570-bp band was detected in all cells, although with different intensity. An identical amount of RNA was used for each cell type.

pH 8) containing 0.25% acetic anhydride. Samples were then dehydrated in ethanol (30-100%) and dried. Hybridization mixtures were added to the slides and incubated overnight at 42°C for radioactive probes and at 70°C for DIG-labeled probes. Posthybridization washes consisted of RNaseA treatment and decreasing concentrations of SSC washes. Radioactively hybridized sections were exposed in NTB2 Emulsion (Eastman Kodak, Rochester, NY) for 7 days, developed photographically, counterstained with Gill's hematoxylin and eosin (0.25% w/v in ethanol), cleared with xylene, and mounted with Permount (Fisher). Sections hybridized with DIG-labeled probes were incubated in 10% sheep serum/TBST (TBS and 1% Tween 20) for 1 h at room temperature and then in alkaline–phosphatase-conjugated anti-digoxigenin antibody (1:1000 dilution) for 2 h. Color development was performed in the dark overnight with 3.4 μl/ml NBT/DMF (Roche) and 3.5 μl/ml BCIP/DMP (Roche) in 0.1 M Tris, pH 9.5/0.1 M NaCl/0.05 M $MgCl_2$. Developed specimens were washed with PBS and mounted with Crystal Mount (Biomeda). Ovaries from both methods were visualized and photographed with AxioCam (Zeiss).

Materials

All other reagents were of the highest quality from Sigma or Calbiochem.

Results

RT-PCR demonstrates the presence of AC3 mRNA in rat and mouse oocytes

An RT-PCR strategy was used to determine whether any of the known adenylyl cyclase genes are expressed in rat and mouse oocytes. Degenerate primers corresponding to the C2 region of the catalytic domain highly conserved in all 9 known transmembrane ACs were used with reverse transcribed RNA prepared from rat oocytes (Fig. 1) (Gautier-Courteille et al., 1998). As controls, cDNAs from granulosa and cumulus cells were also amplified. A fragment of the appropriate size was amplified in the three different experiments performed with oocyte RNA, suggesting that an adenylyl cyclase mRNA is indeed expressed in the female gamete. In order to identify the AC gene expressed, the fragments were subcloned into TOPO vector, and 21 individual clones from 3 amplifications on different oocyte RNA preparations were sequenced. From the sequences of the clones amplified from the rat oocyte mRNA, 17 clones encoded AC3, 2 AC9 and only 1 encoded AC6 and AC7, respectively (Fig. 2). Conversely, AC6 and AC9 mRNAs were the most frequent mRNAs amplified in granulosa and cumulus cells. Therefore, this analysis indicated that AC3 is an abundant mRNA in rat oocytes, whereas other AC genes are predominantly expressed in the somatic compartment of the rat ovary.

To confirm the expression of a cyclase in rat and mouse oocytes, AC3-specific primers were used to amplify oocyte mRNA from the two species (Gautier-Courteille et al., 1998). In agreement with the amplification using the degenerate primers, AC3 mRNA could be detected in both rat and mouse oocytes (Fig. 3). A subsequent experiment using degenerate primers confirmed that mRNAs for AC1 and AC9 are the forms most frequently amplified in the mouse, suggesting that AC3 mRNA is predominant in rat oocyte but not in mouse oocytes (data not shown). It should be pointed out that EST analysis from a mouse unfertilized egg library suggests the expression of AC9. Thus, overlapping sets of AC genes may be expressed in the oocytes from rat and mouse.

Detection of the AC3 mRNA in rodent oocytes by in situ hybridization

Because RT-PCR amplified AC3 from both the oocyte and granulosa cell mRNA, the site of expression of this mRNA in the ovary was further investigated by in situ hybridization.

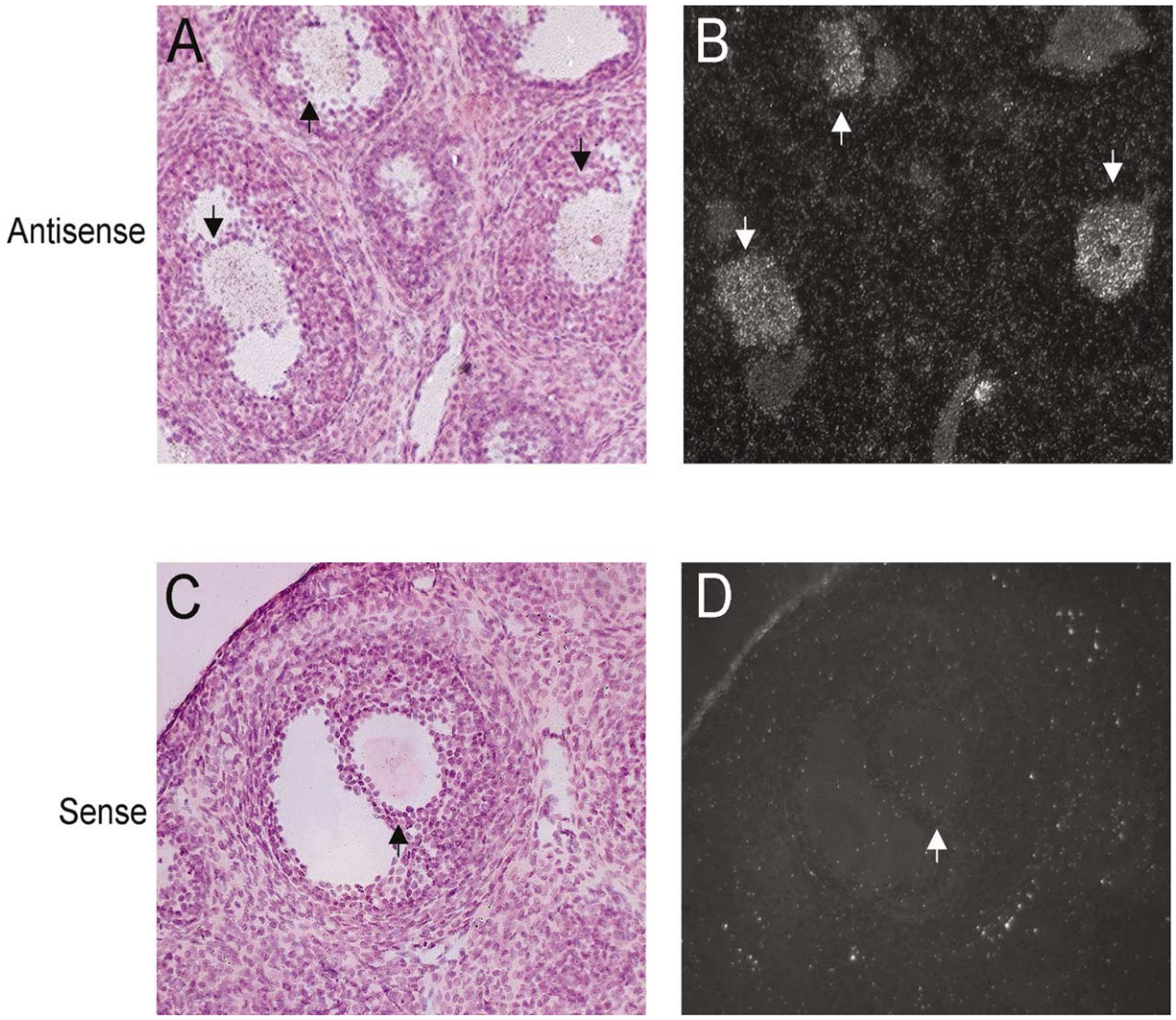


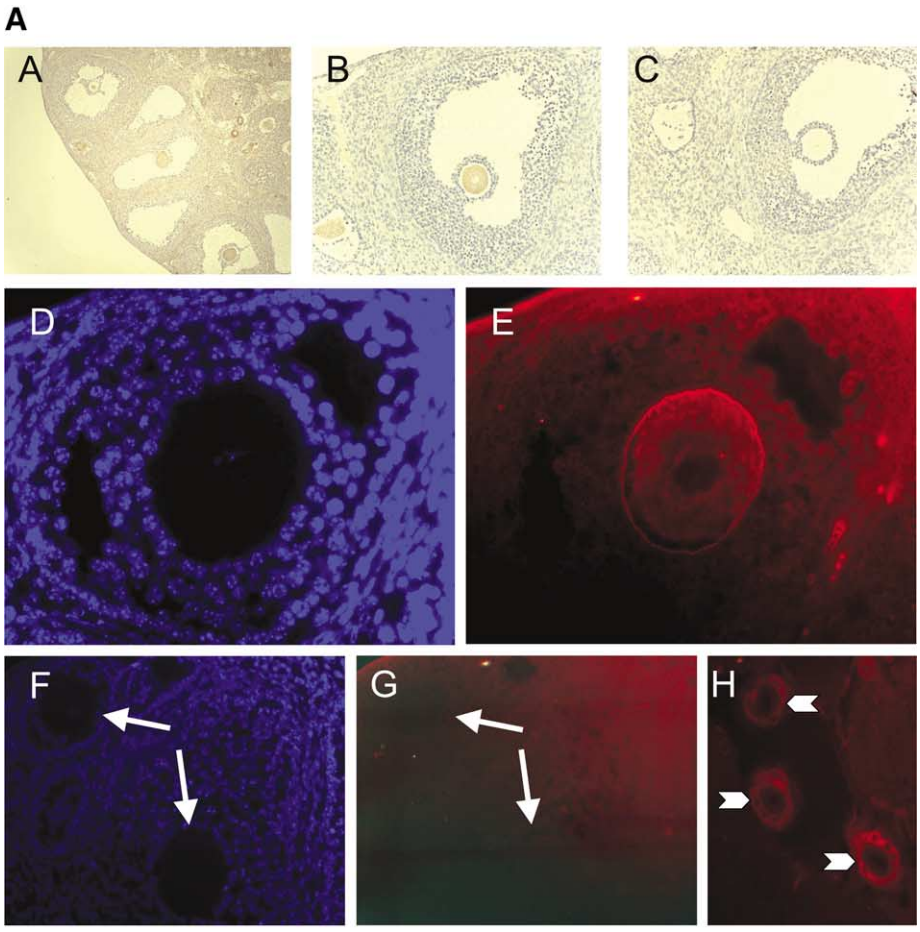
Fig. 4. Detection of AC3 expression in the rat ovary using in situ hybridization. Ovaries were excised from PMSG-stimulated rats and processed as detailed in Materials and methods. Sections were hybridized with antisense and sense AC3 cRNA, processed in an identical manner, and exposed for 7 days. Signal was prominent in oocytes from immature and mature follicles. Some signal was also present in the interstitium. Minimal signal above background was observed in granulosa cells (200× magnification).

Sections of rat ovary were used for in situ hybridization by using a radioactive (Fig. 4) or digoxigenin-labeled (data not shown) AC3-specific probe. In both cases, the predominant signal with the antisense probe was localized on the oocytes. When follicles at different stages of development were evaluated, signal was moderate on oocytes from primary and secondary follicle and increased to a maximum in preantral/early antral follicles (data not shown). Additional minor diffuse signal was present in the granulosa cells and in the interstitium of the ovary, consistent with the RT-PCR data (see Fig. 3). The use of a sense AC3 probe demonstrated a minor diffuse signal throughout the section of the ovary.

Immunocytochemistry/immunofluorescence analysis of the expression of the AC3 protein in the rat follicle

To confirm that an AC3 protein is indeed expressed in rat oocytes, an antibody raised against the carboxyl terminus

Fig. 5. Immunocytochemical and immunofluorescence localization of AC3 in the rat (A) and mouse (B) ovary. (A) Immunohistochemical localization of AC3 in immature rat ovaries stimulated with PMSG at a magnification of 100× (A) and 200× (B, C). In (C), the primary antibody was replaced by nonimmune IgG. In (E, G, H), the localization of AC3 in rat ovaries was followed by using FITC-labeled secondary antibodies [magnification of 630× (D, E) and 200× (F–H)]. (D, F) The nuclei stained with DAPI; the same sections as (E) and (G). In (G), an excess of blocking peptide was added on the section prior to the incubation with the primary antibody. The location of oocytes is indicated by arrows in (F) and (G). In (H), labeled vessels are indicated by thick arrows. (B) Localization of AC3 in mouse ovaries by immunofluorescence (630×). (A) Wild type ovary. (B) Ovary from AC3 knockout mouse. (C, D) Corresponding staining with DAPI. ACIII antibody was visualized with Texas Red anti-rabbit secondary IgG, and the nuclei were stained with DAPI. Oocytes are indicated by arrows.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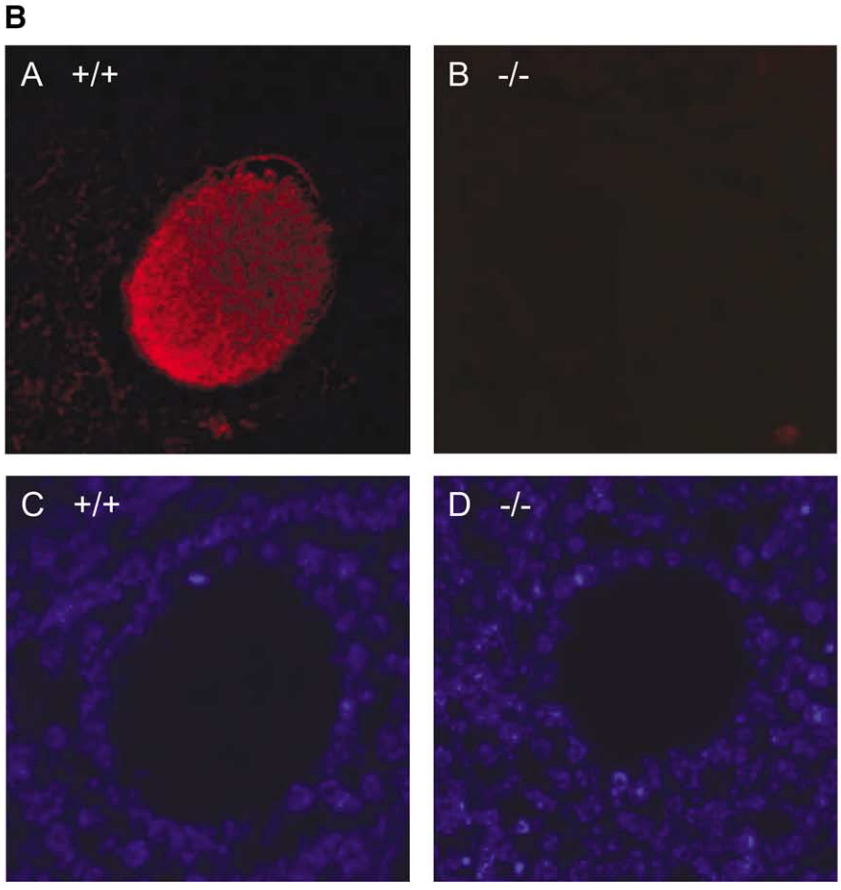
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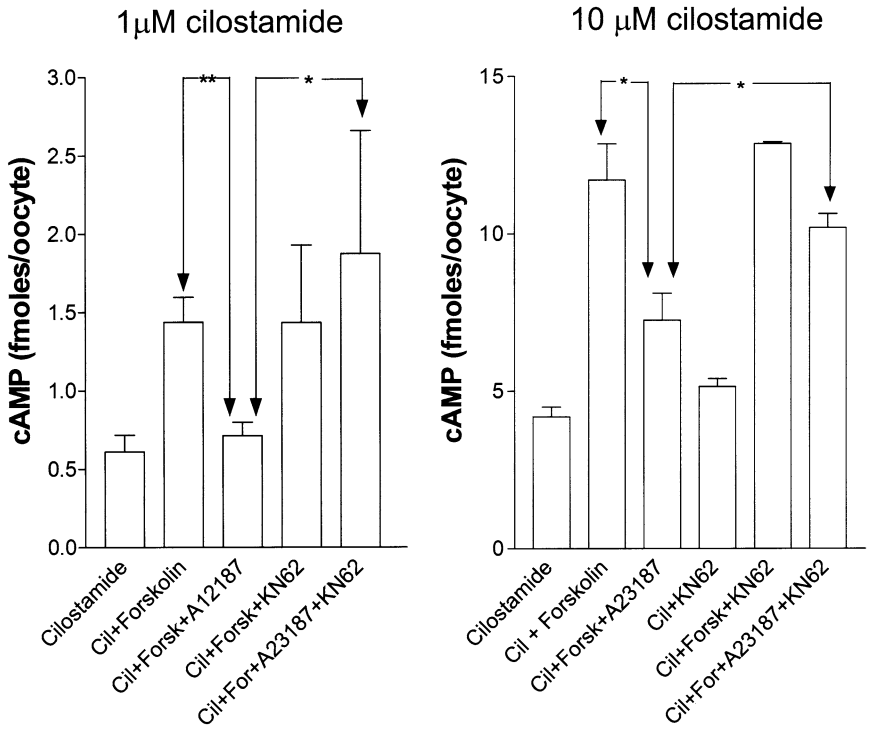

Fig. 6. Effect of intracellular Ca^{2+} on cAMP accumulation in denuded rat oocytes. Denuded rat oocytes were incubated at 37°C for 30 min in complete medium supplemented with 4 mM Ca^{2+} and 1 or 10 μM cilostamide. At the end of the incubation, oocytes were quickly frozen and thawed, and cyclic AMP levels were measured by radioimmunoassay. Data are the means ± SEM of three experiments performed at least with duplicate samples. *, $P < 0.05$; **, $P < 0.01$.

region specific for this cyclase was used for immunolocalization. Using this antibody in the ovary, immunoreactivity was present predominantly in rat and mouse oocytes (Fig. 5A and B) at all stages of development. This immunoreactivity was specific because the signal was absent when omitting the primary antibody or was greatly reduced when the primary antibody was initially adsorbed to the peptide used for immunization (Fig. 5A).

The localization of AC3 on the plasma membrane of the oocytes was further confirmed when fluorescent secondary antibodies were used. A well-defined signal on the oocyte plasma membrane was observed together with some fluorescence in the cytoplasm. The signal was again specific since it was absent when antibodies were adsorbed to the peptide. The specificity was further confirmed by the finding that no signal was observed in mouse oocytes from *Adcy3*$^{-/-}$ mice (Fig. 5B). In agreement with the in situ hybridization data, the surrounding cumulus cells show only minimal staining, whereas some specific staining was evident in the interstitium of the ovary where the vessel wall was positive. Expression of AC3 in endothelial cells of blood vessels has been reported (Wong et al., 2001).

Cyclic AMP regulation in rat oocytes is consistent with the expression of a cyclase with the properties of AC3

The properties of AC3 have been extensively studied (Hanoune and Defer, 2001; Wei et al., 1996; Wei et al., 1998). This cyclase is activated by Gs protein and is inhibited by an increase in intracellular Ca^{2+}. However, unlike AC1 and AC8, where Ca^{2+}-calmodulin directly activates the enzymes, the Ca^{2+} regulation of AC3 is indirect, being mediated by CaM kinase II phosphorylation (Wei et al., 1996). Therefore, if an adenylyl cyclase with the properties of AC3 was indeed expressed in the oocytes, cAMP levels should be increased by exposing the oocytes to forskolin and the effect suppressed by raising intraoocyte Ca^{2+} levels. To test this possibility, cAMP accumulation was determined in oocytes after incubation with forskolin in the absence or presence of the ionophore A23187. In these experiments, two different concentrations of the PDE3 inhibitor cilostamide were used; we have previously shown that PDE3 is the major PDE expressed in the rat oocytes. Under both conditions, forskolin stimulated cAMP levels approximately two- to threefold (Fig. 6). More importantly, increasing Ca^{2+} in the oocytes by A23187 completely abolished the forskolin effect, and the inhibitory effect of the ionophore was prevented by a CaMKII inhibitor KN62.

The altered levels in cAMP using forskolin and A23187 should impact the rate of progression of the oocytes through the cell cycle. Indeed, we observed that in the presence of forskolin the resumption of meiosis of rat oocytes was delayed by approximately 2 h (data not shown). Conversely, the effect of the ionophore could not be tested in this model because this compound by itself blocks oocyte maturation,

probably due to additional effects of the large increase in Ca^{2+} in the oocyte.

Defect in meiotic arrest in mouse oocytes deficient in AC3

The data reported above indicate that an active AC3 contributes to cAMP levels in rat oocytes. This AC mRNA and protein are also expressed in mouse oocytes, even though it may not be the predominant form because mRNAs for other ACs are present, as detected by the RT-PCR strategy. *Adcy3*-deficient mice have been generated, providing a model in which to investigate meiotic resumption in the absence of AC3 expression (Wong et al., 2000). However, most of these mice die soon after birth because of an olfactory deficit and other less defined phenotypes (Wong et al., 2000). This poor viability has largely impeded the investigation of the impact of this gene on fertility. Nevertheless, it was noticed that the few females that reached maturity were able to mate normally but had reduced fertility and fecundity (Wong et al., 2000), a finding confirmed in our laboratory.

In our colony, we have been able to raise five female homozygous null for *Adcy3* to a stage where oocyte maturation could be studied. By day 24, the body weight of the *Adcy3* null mice was comparable with that of wild type littermates (WT = 12.6 ± 1.2 g *Adcy3* null = 11.1 ± 1.3 g Mean ± S.D., $P > 0.05$). The ovaries from these mice are grossly normal (Fig. 7), with a normal complement of follicles at different stages of development. However, it was noted that an unusual number of oocytes in follicles at early antral stage had no GV, often with a discernible spindle and condensed chromosomes in the equatorial plate (Fig. 7B) In addition, some follicles contained a multinucleated structure that resembled embryos, which could be the result of parthenogenic activation of the oocytes or nuclear fragmentation after the first meiotic division (Fig. 7C). Morphological quantitation of the number of oocytes at GVBD, MII, or further stages of development indicated that more than 50% of the oocytes in the *Adcy3* null mouse have resumed meiosis (Fig. 7D), whereas only 10-15% of the wild type oocytes show signs of meiotic resumption (% of oocytes without GV: WT = 12.0 ± 2.5, *Adcy3* null 55.1 ± 4.2, Mean ± SEM, $P < 0.0001$). Moreover, oocytes isolated from three homozygous null PMSG-treated females matured in vitro at a rate which is slightly but consistently faster than control oocytes from littermates ($P < 0.05$) (Fig. 8). Thus, it is possible that precocious resumption of meiosis may contribute to the reduced fertility phenotype suggested for these mice.

Discussion

The intracellular concentration of cAMP is thought to play a critical role in maintaining the meiotic arrest in competent mammalian oocytes (Conti et al., 2002). With the present study, we provide evidence that oocytes are able to synthesize cAMP. Adenylyl cyclase mRNAs are detected in rodent oocytes, with *Adcy3* being the predominant gene expressed in the rat, whereas *Adcy1*, *Adcy9*, and *Adcy3* are expressed in the mouse gamete. In rat oocytes, a cyclase with the properties of AC3 is responsible for cAMP accumulation in the oocytes. Finally, mouse oocytes deficient in AC3 protein display signs of inefficient or leaky meiotic arrest both in vivo and in vitro. Collectively, these data demonstrate that oocytes are able to produce cAMP and that the oocyte meiotic arrest may be controlled via regulation of this cyclase.

Our data show that AC3 mRNA and protein are expressed in rat and mouse oocytes. This conclusion is based on the RT-PCR, on the in situ hybridization data, and on the immunocytochemistry of rat and mouse ovaries. More importantly, we provide functional evidence that forskolin increases cAMP in denuded oocytes and that this stimulation is blocked by the ionophore A23187. These findings are consistent with the hypothesis that the cyclase expressed in the oocytes has the functional properties of AC3. In several systems, it has been reported that AC3 is inhibited by Ca^{2+} via activation of CaM kinase II. Indeed, we found that inhibition of cAMP accumulation by A23187 is blocked by a CaM K inhibitor KN-62. Thus, an active AC3 is expressed in rat and mouse oocytes. A difference between the two species is that AC3 is the predominant form expressed in the rat, whereas other forms coexist with AC3 in the mouse oocytes.

That oocytes are able to synthesize cAMP was further indicated by the finding that incubation of denuded oocytes with PDE3 inhibitors caused a progressive increase in cAMP. On the basis of our previous findings in intact or broken cell preparations (Richard et al., 2001), it was calculated that the oocyte expressed enough PDE3 activity to deplete the oocyte cAMP pool (1-2 fmole/oocyte) in less than 1 min after isolation. Because cAMP is still measurable in denuded oocytes 1-2 h after incubation in the absence of PDE inhibitors, we surmise that an adenylyl cyclase in oocytes is actively producing cAMP at rates sufficient to balance the degradation by PDE3A. In the same vein, cAMP levels increase in denuded oocytes when PDE3 inhibitors are added because of continuing synthesis. It is unlikely that this increase in cAMP is due to remnants of cumulus cells attached to the surface of the oocyte for the following reasons. We have previously reported that PDE3A is not detectable in granulosa cells and that therefore cAMP levels should not be affected by PDE3-specific inhibitors (Tsafriri et al., 1996). More importantly, AC3 mRNA is present at low levels in rat granulosa cells, and AC3 protein is barely detectable by immunocytochemistry. Therefore, it is highly unlikely that inhibition of cAMP accumulation by A23187 is due to an effect on these latter cells, even if the preparation of denuded oocytes does fail to remove all traces of cumulus cell membranes. Collectively, these findings strongly support the conclusion that rat oo-

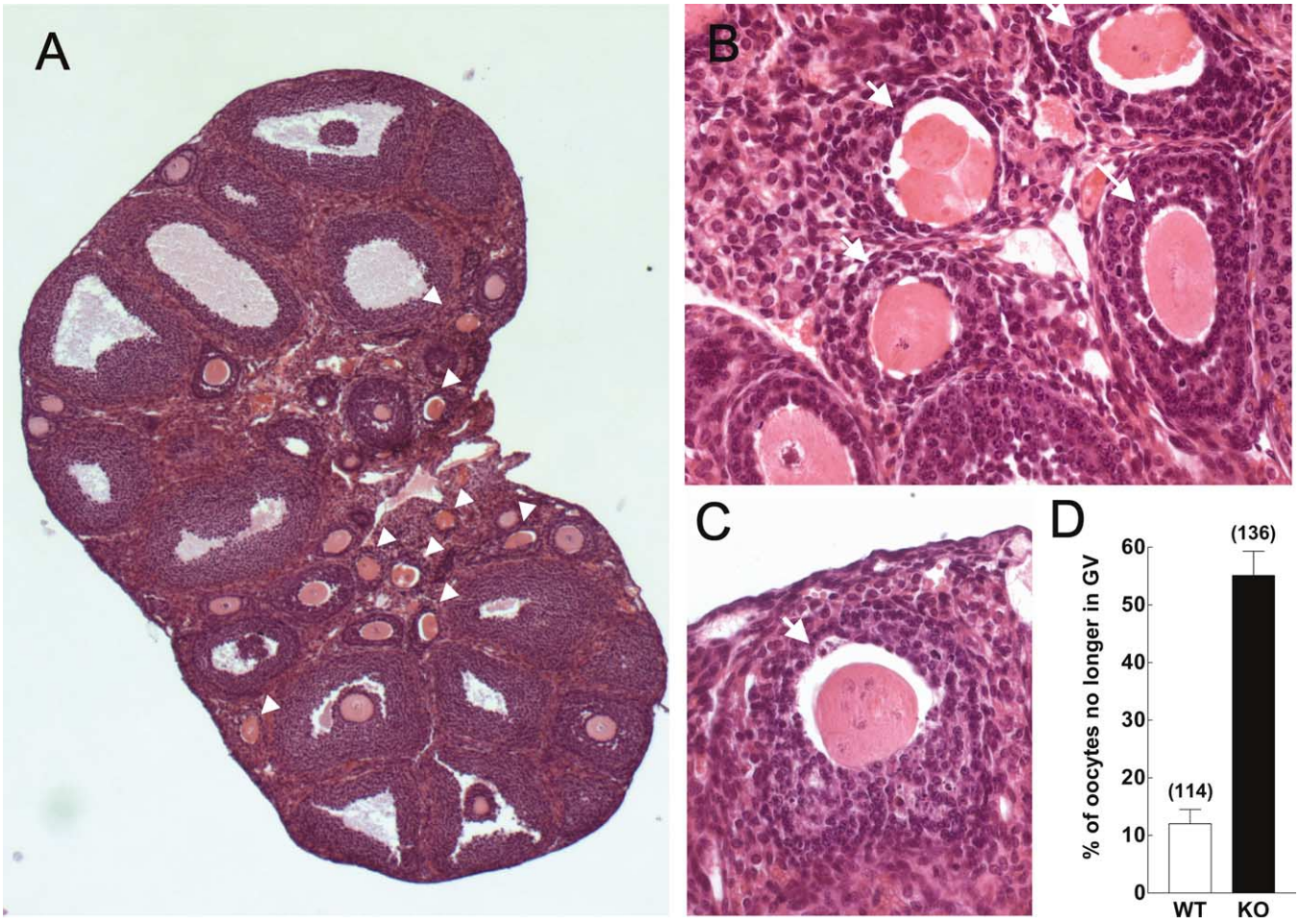

Fig. 7. Morphology of the ovary from immature, PMSG-treated mice deficient in *Adcy3*. Ovaries were processed for histology and sections were stained with hematoxylin and eosin. A median section through the ovary is reported in (A) (magnification 23×). (B, C) Higher magnification of follicles containing degenerating or abnormal oocytes. Oocytes no longer in GV are indicated by white arrows. (D) Ovarian sections from wild type and *Adcy3* null mice were used to score oocytes in GV and oocytes that had resumed meiosis. The latter group included oocytes without distinguishable nucleolus and/or with meiotic spindle and polar body, or multinucleated embryonic structures. Bars represent the % of oocytes not in GV (Mean + SEM, $N = 3$). The number of oocytes scored is reported above the columns. Differences between the two genotypes were significant ($P < 0.0001$).

cytes express AC3 mRNA and protein which actively synthesizes cAMP.

Our data are consistent and extend the recent observation that injection of Gs-inactivating antibodies causes resumption of meiosis in follicle-enclosed oocytes (Mehlmann et al., 2002). These studies assume that Gs inhibition causes a decrease in activity of a cyclase expressed in the oocytes. Our findings strongly indicate that AC3 or related cyclases are the enzymes downstream of Gs in the mature gamete. It remains to be determined whether the third component of the membrane signal transduction machinery, a G-protein coupled receptor, is present in the oocyte and is signaling to maintain an active Gs and adenylyl cyclase.

A puzzling finding is that the forskolin response of oocytes in terms of cAMP accumulation is small when compared with that usually detected in somatic cells. It is also small compared with the almost 10-fold increase in oocyte cAMP following addition of the PDE3 inhibitor. These observations argue that a balance between synthesis and degradation of cAMP is present in the oocytes but that this equilibrium is different from somatic cells, where pharmacological stimulation of cAMP synthesis is more effective than inhibition of degradation in controlling the steady state. In the oocyte, cAMP turnover must be very rapid with a high basal level of cyclase activity balanced by a very active phosphodiesterase, and inhibition of the activity of this degrading enzyme has a major impact on cAMP steady state.

Analysis of the phenotype of the AC3-null mice shows that spontaneous maturation in denuded oocytes occurs at a rate which is slightly but significantly faster than controls. Furthermore, the morphological analysis of the ovary of the *Adcy3*-deficient mice revealed that more than 50% of oocytes have resumed meiosis. These data suggest that a defect in meiotic arrest may follow ablation of *Adcy3*. It was also noticed that these degenerating oocytes are prevalent in preantral or early antral follicles. These oocytes must have acquired meiotic competence and have probably undergone

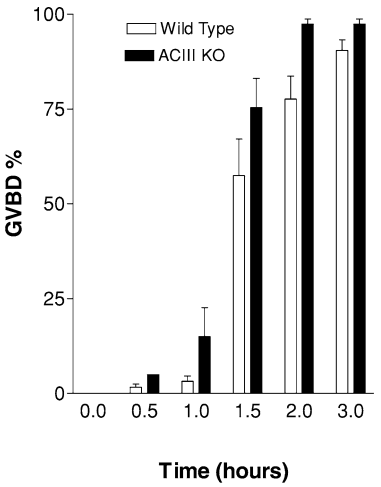

Fig. 8. Rate of spontaneous maturation of mouse oocytes from AC3-deficient immature mice. *Adcy3*-null mice and wild-type littermates were injected with 5 IU of PMSG. After 45 h, ovaries were punctured to release oocytes. Oocytes from one or two ovaries were collected in Leibovitz's L-15 medium supplemented with 5% fetal calf serum and 100 μg/ml penicillin and streptomycin and denuded by pipetting through a small-bore polycarbonate tip. They were scored every 30 min for up to 3 h by Hoffman interference for signs of morphological maturation. Data are expressed in terms of % of germinal vesicle breakdown (GVBD) as the mean ± SEM of at least 3 separate experiments for each genotype, with 119 oocytes for wild type and 87 oocytes for the AC3-null mice. The time course of $Adcy^{-/-}$ oocyte maturation was significantly different from that of wild type oocyte ($P = 0.015$).

precocious maturation during follicles and increased in development. AC3 mRNA and protein were detected in oocytes in secondary follicles and increases in preantral follicles, suggesting that these stages may be particularly sensitive to ablation of *Adcy3*. This finding is consistent with the hypothesis that AC3 contributes to the generation of cAMP in the oocytes and to the maintenance of meiotic arrest. The phenotype does not have complete penetrance probably because, unlike that observed in the rat, other adenylyl cyclases, whose mRNA were detected in mouse oocytes, probably compensate to some extent for the loss of AC3 expression. At present, we cannot completely exclude the possibility that some of the phenotypes observed may be due to pleiotropic effects that follow ablation of this gene or to a defect in the granulosa cell function, as low levels of AC3 mRNA were detected in these cells.

The finding that an oocyte expresses an adenylyl cyclase that is inhibited by a Ca^{2+}-dependent phosphorylation opens the possibility that this regulation may participate in signaling meiotic resumption. An increase in oocyte intracellular Ca^{2+} would decrease AC3 activity and contribute, together with the observed PDE3A activation, to the decrease in cAMP levels necessary for meiotic resumption. Pharmacological manipulations indicate that changes in intracellular Ca^{2+} and CaM-dependent events may be required for oocyte maturation (Bornslaeger et al., 1984; De Felici et al., 1991; Homa et al., 1993; Su et al., 1999). However, conflicting results have been reported as to whether an increase in intraoocyte Ca^{2+} indeed occurs after gonadotropin stimulation of the follicle (Mattioli et al., 1998; Webb et al., 2002). Previous work from Eppig and collaborators investigated the effect of CaM kinase II inhibitors on oocyte maturation in the mouse (Su and Eppig, 2002). This study reached the conclusion that CaM kinase II is required for gonadotropin-activated maturation, for the transition between metaphase I to anaphase, and for polar body extrusion. However, CaM kinase II inhibitors did not have an effect on GVBD during spontaneous maturation of denuded or cumulus-enclosed oocytes. In view of these findings, further assessment of a CaMKII and AC3 involvement in maturation is warranted.

The discovery that AC3 is expressed in oocytes also may be relevant to the control of activation of MII-arrested oocytes. It is well established that eggs are activated following sperm penetration and that an incompletely defined cascade of events leads to a spike of intracellular Ca^{2+} followed by oscillations (Runft et al., 2002). It has been also demonstrated that CaM kinase II is localized in the MII spindle (Johnson et al., 1998) and that its activation plays a critical role for completion of meiosis (Runft et al., 2002). Because AC3 is inhibited by CaM kinase II, it is feasible that the activation of this kinase impacts the activity of AC3 and therefore the cAMP concentration in the eggs. The consequent reduction in cAMP may be important for metaphase/anaphase transition since the inactivation of PKA is associated with this transition (Kotani et al., 1998).

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