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Identification of PDE9 as a cGMP-specific phosphodiesterase in germinal vesicle oocytes: A proposed role in the resumption of meiosis

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Abstract

Objective—To identify a cGMP-specific phosphodiesterase (PDE) in non-human primate germinal vesicle (GV) oocytes and establish a proposed effect on oocyte maturation through preliminary experiments in mouse GV oocytes.

Design—Controlled non-human primate and rodent experiments.

Setting—Academic research institution.

Animals—Rhesus macaques and B6/129F1 mice.

Interventions—Rhesus macaques were stimulated with FSH to collect GV oocytes and cumulus for gene expression analysis. Female mice were stimulated with PMSG to collect GV oocytes.

Main Outcome Measures—PDE transcript expression in primate GV oocytes and cumulus cells. Fluorescence polarization (FP) measurements of PDE3A activity. Spontaneous resumption of meiosis in mouse GV oocytes.

Results—Five PDE transcripts were detected in Rhesus GV oocytes, only *PDE9A* was cGMP-specific. FP assays indicated cGMP has an inhibitory effect on PDE3A while the PDE9 inhibitor, BAY73-6691, did not. Similarly, BAY73-6691, had little effect on preventing spontaneous maturation in oocytes, but did augment the inhibitory effects of cGMP. Inclusion of 0μM (control), 10μM, 100μM, and 1 mM BAY73-6691 significantly increased the proportion of mouse oocytes maintaining GV arrest in the presence of the cGMP analog 8-Br-cGMP at: 100μM (8.8%, 11.4%, 18.8%, and 28%), 500μM (21.1%, 38.1%, 74.5%,and 66.5%), and 1 mM (57.8%, 74.5%, 93.9%, and 94.0%) respectively, when $P<0.05$.

Conclusions—PDE9 is a cGMP-specific hydrolyzing enzyme present in primate oocytes, and PDE9 antagonists augment the inhibitory effect of cGMP during spontaneous in vitro maturation of GV mouse oocytes.

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Keywords

oocyte; meiosis; phosphodiesterase 9A; cGMP; phosphodiesterase 3A

Introduction

Oocyte maturation is an integral part of the reproductive cycle and critical for normal fertility in women. Oocytes remain arrested at the germinal vesicle stage (GV) of prophase I by maintaining elevated levels of cyclic AMP (cAMP) (1, 2). The intra-oocyte cAMP levels drop dramatically following the LH surge, or upon the release of the oocyte from the follicular environment, due to decreased synthesis and increased hydrolysis of cAMP by selected cyclic nucleotide phosphodiesterases (PDEs). PDEs are a class of enzymes that hydrolyze the second messengers cAMP and cGMP (3). There are 11 different families of PDEs characterized by their relative affinities and binding capacities for each second messenger (4). Within the oocyte, PDE3A has been identified as the primary cAMP-hydrolyzing phosphodiesterase (5–9). Inhibition of PDE3 has been shown to prevent both the spontaneous and gonadotropin-induced maturation of oocytes in a number of species, including human and non-human primates (6, 10–17).

Recent studies have shown that cGMP plays an important role in maintaining the meiotic arrest state of the GV oocyte suppressing the activity of PDE3A (18). cGMP is produced by the granulosa cells and diffuses into the oocyte via gap junctions (18–21). When the LH surge occurs, gap junctions close, cutting off the supply of cGMP to the oocyte. The concentration of cGMP in the oocyte gradually declines which then permits PDE3A to hydrolyze cAMP into 5'AMP, leading to meiotic resumption and the production of a fertilizable egg (5, 22–25). It is likely that a PDE(s) is present in the oocyte which targets residual cGMP for degradation after gap junction closure, however the specific PDE has until now not been identified. Inhibitors for cGMP-specific PDEs in the oocyte could provide a targeted approach to prevent hydrolysis of cGMP that would in turn block down-stream degradation of cAMP and prolong oocyte meiotic arrest, even after ovulation.

Pharmacological inhibition of PDE3 has been shown to inhibit oocyte maturation and prevent pregnancy in rhesus macaques (26). However, unfavorable side effects and inconsistent bioavailability of currently available PDE3 inhibitors suggest the need for further investigation of alternate contraceptive strategies (26). We hypothesized that suppressing cGMP-targeting PDE(s) with specific inhibitors will maintain elevated intra-oocyte cGMP levels and block (or delay) PDE3A-induced cAMP degradation preventing timely resumption of meiosis and circumventing the need to target PDE3A directly. Herein we report the expression pattern of PDE genes in the primate follicle and the identification of a cGMP-targeting enzyme in the monkey GV oocyte. Additionally we evaluated the function of this PDE by assessing the effect of its inhibitor on recombinant PDE3A activity in a fluorescence polarization assay and on spontaneous mouse oocyte maturation, either alone or in combination with exogenous cGMP.

Materials and Methods

Animal use

All animal protocols and procedures were performed after approval from, and in strict accordance to the Oregon Health & Science University Institutional Animal Care and Use Committee and followed the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

Collection of rhesus monkey germinal vesicle oocytes and granulosa cells

Ovary aspiration and collection of GV oocytes in the Rhesus monkey (*Macaca mulatta*) has been previously described (27). Briefly, antral follicles were aspirated following a standard controlled ovary stimulation protocol modified to exclude administration of human chorionic gonadotropin (hCG) (28). GV oocytes were aspirated into warm TL Hepes medium (Lonza Walkersville Inc., Walkersville, Maryland, USA) supplemented with 5 IU/ml heparin (MP Biomedicals, Solon, Ohio, USA). Total volume of the aspirate was adjusted to 10 ml with fresh TL Hepes and 3 mg of hyaluronidase (Sigma-Aldrich, St. Louis, Missouri, USA) was then added. The aspirate was filtered through a 70-μm nylon cell strainer (BD Biosciences, Bedford, Massachusetts, USA), and remaining debris in the filter cup was collected with 10 ml fresh TL Hepes into a 60 mm × 15 mm petri dish. GV oocytes were collected and prepared for RNA isolation by removing cumulus cells with repeated pipetting through a fine bore glass capillary needle.

Granulosa cells from the filtered aspirate were collected into a 15-ml conical tube and pelleted by centrifugation at 3000 × g for 2 minutes. The cell pellet was resuspended in 500 μl warm DMEM cell culture medium (Invitrogen, Carlsbad, California, USA) and layered on top of a 3-ml 30% Percoll/DMEM mixture in a 15-ml conical tube. The suspension was centrifuged at 3000 × g for 30 minutes to remove the red blood cells from the granulosa cell mixture, and the top layer with the purified granulosa cells was washed in 10 ml of fresh DMEM and centrifuged for an additional 3 minutes at 3000 × g. After removal of the supernatant, the granulosa cell pellet was considered ready for RNA isolation.

Collection and preparation of mouse germinal vesicle oocytes

B6D2F1 (BDF-1) mice (Charles River Laboratories, Wilmington, Massachusetts, USA) at 3–6 weeks of age were stimulated with 5 IU of PMSG (Harbor UCLA, Torrance, California, USA), administered intraperitoneal to promote follicle growth. After 48 hours, ovaries were collected into warm M2 medium (Millipore, Billerica, Massachusetts, USA) containing 200 μM 3-Isobuty-methylxanthine (IBMX; Sigma-Aldrich), and follicles were repeatedly punctured with a 27G needle to release the oocytes. Cumulus granulosa cells were removed by repeated pipetting through a fine bore glass needle, and cumulus-free, GV-intact oocytes were washed in fresh KSOM medium (Millipore) without IBMX for additional culture experiments.

RNA isolation, RT-PCR, and quantitative PCR (qPCR)

RNA from rhesus monkey GV oocytes and granulosa cells was isolated following the manufacturer's protocol for the Absolutely RNA™ Nanoprep Kit (Agilent Technologies, Inc., Wilmington, Delaware, USA). Isolated RNA samples were treated with RNase-free DNase (Promega, Madison, Wisconsin, USA) and eluted with 11 μl of nuclease-free water. The eluate was directly amplified with Superscript III reverse transcriptase and random hexamer primers (Invitrogen) to produce cDNA. The quality of cDNA for each sample was verified by PCR amplification of the housekeeping gene peptidylprolyl isomerase A (PPIA) using HotStar taq polymerase (QIAGEN, Valencia, California, USA); the product was then visualized on a 2% ethidium bromide-stained agarose gel. All RT-PCR primers were designed using Primer 3 software (Sigma-Aldrich). Although the zona pellucida was not removed from the oocytes prior to RNA isolation, all attached cumulus cells were removed to prevent transcript contamination in the samples. As cumulus-oocyte heteromeric gap junctions do not permit permeability of molecules greater than 0.1 kDa (PDE transcript average size ~600 kDa), it is expected trans-zonal projections would not be a source of RNA contamination (29).

Prior to qPCR amplification, the concentration for each cDNA sample was measured using a Nanodrop ND-1000, and nuclease-free water was used for all required dilutions. The standard curve method was implemented to detect 19 specific PDE genes in the pre-ovulatory rhesus monkey GV oocyte or the associated granulosa cells. Ribosomal protein S-10 (RPS-10) and 18s ribosomal RNA (18s-rRNA) were used as the endogenous control genes to establish expression levels of the PDE messages in granulosa cells and GV oocytes, respectively. qPCR primers were designed with an on-line program from Biosearch Technologies (Novato, California, USA) and ordered through Sigma-Aldrich (Supplemental Table 1). qPCR was performed in 1x Fast SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA), 20 ng of cDNA and 80 nM of each primer in a 20-μl total volume reaction. Amplification and detection were performed with a 7500 Fast Real-Time PCR system (Applied Biosystems) over 50 cycles. For each gene, a standard curve was performed to assess the quality of the reaction and quantify relative expression levels of all PDE genes compared to the endogenous control.

PDE3A Inhibition Assay

Prior to mouse oocyte culture experiments, the direct inhibitory effects on PDE3A activity by 8-Br-cGMP and BAY 73-6691 were measured with a cell-free fluorescence polarization (FP) assay. FP assays corrects for rotation of the fluorophore, induced by absorbance of polarized light and interaction with a ligand, by measuring fluorescence intensity both parallel and perpendicular to the detectors. This method provides more accurate measurement of fluorescent biochemical activity than standard single-read assays. Using a PDE3A FP Assay Kit (Bioscience, San Diego, California, USA),the protocol was followed per manufacturer's suggestions to determine the percent decrease of PDE3A activity achieved in the presence of increasing concentrations of either compound. Inhibitory activity of 8-Br-cGMP was measured at 1 μM, 10 μM, 100 μM, 1 mM, and 10 mM and BAY 73-6691 at 1 μM, 10 μM, 100 μM, and 1 mM. Five replicates were performed for each compound series over 3 different assay kits. Fluorescent cAMP was incubated with 400 pg of human PDE3A (92% homologous to mouse PDE3A) and different concentrations of either 8-Br-cGMP or BAY 73-6691 in a 96-well microtiter plate in triplicate. PDE3A activity was indicated by generation of fluorescent nucleotide monophosphates, a by-product of PDE3A induced cAMP hydrolysis; inhibition of PDE3A reduces the concentration of the monophosphates available for measurement. Fluorescence polarization measurements (mP) were obtained at 520 nm with a Molecular Devices SpectaMax M5 microplate reader. Data collected were then used to define a range of concentrations to evaluate during mouse oocyte culture and validate that dose response effects observed in these experiments were due to PDE3A inhibition.

Mouse GV oocyte co-culture

All culture experiments were carried out in KSOM medium at 37°C in a humidified 5% CO_2 incubator for 20–22 hours. A membrane-permeable cGMP analog, 8-Br-cGMP (Sigma-Aldrich), was used as an exogenous cGMP source and dissolved in KSOM to make a 100 mM stock solution. The stock solution was diluted to 100 μM, 500 μM, 1 mM or 10 mM in KSOM medium and used for mouse GV oocyte culture. BAY 73-6691 (Sigma-Aldrich), a membrane permeable, specific PDE9 inhibitor, was dissolved in DMSO to make a 100 mM stock solution. The BAY 73-6691 stock solution was also diluted in KSOM medium at 10 μM, 100 μM, or 1 mM for oocyte incubation. Co-culture experiments with 8-Br-cGMP and BAY 73-6691 together at varying concentrations were also performed. Each experiment included a control group of oocytes incubated in KSOM without supplementation. The criterion for spontaneous oocyte maturation was the disappearance of a visible nucleus, i.e., germinal vesicle breakdown (GVBD).

Statistical analysis

All qPCR data sets represent a mean value based on three experimental replicates, over three different biological samples. Expression values for these data are graphically displayed as a relative fold change compared to an endogenous control gene (Figure 1). The first standard deviation is indicated; means were evaluated using One Way ANOVA and pair wise comparisons were performed with the Student-Newman-Keuls method with $P < 0.001$ considered significant to adjust for multiple comparisons.

Data from the fluorescence polarization measurements were analyzed for the Z'-factor to assess data quality and variability (30). Z'-factor values between 0.5 and 1.0 are considered excellent (good quality, repeatable data) while data with Z'-factor values below 0.5 are considered less reliable. Significant changes in mP values were determined by One Way ANOVA followed by posthoc comparisons with the Student-Newman-Keuls test where $P < 0.001$. The first standard deviation is indicated for both compounds evaluated (Figure 2).

A total of 32 mice were used to collect oocyte GV retention data over 5–6 experimental replicates. The SD is indicated for all the mean values of the proportions. The statistical significance was determined by Chi Square analysis with a criterion of $P < 0.05$.

Results

Cellular distribution of PDE transcripts in rhesus monkey antral follicles

Rhesus monkey GV oocyte and granulosa cells collected from preovulatory (no LH) antral follicles were analyzed by qPCR to identify the PDE genes actively expressed at this stage of development. Of the six genes in the PDE6 family (PDE6A, PDE6B, PDE6C, PDE6D, PDE6G, PDE6H), only PDE6A was selected for detection by qPCR. In preliminary experiments using macaque ovarian tissue, standard RT-PCR analysis failed to detect any of the remaining 5 genes in the PDE6 family (data not shown). Among the 19 PDE genes assayed in the GV oocyte, only five transcripts were detected: *PDE1C, PDE3A, PDE8A, PDE8B*, and *PDE9A* (Figure 1A). Of these only PDE9A is cGMP-specific. The remaining oocyte-localizing PDE transcripts predominantly target cAMP (*PDE8A/B*) or have an affinity for both cAMP and cGMP (*PDE1C* and *PDE3A*). There were no statistically significant differences in the expression levels of these five oocyte-localizing transcripts.

In contrast, all of the PDE genes assayed were detected in granulosa cells (Figure 1B) with the exception of *PDE11A*. *PDE4C*, which primarily targets cAMP for degradation, was the most abundantly expressed and displayed a significant 1.6–2.3 fold increase ($P < 0.001$) in signal compared to all other transcripts.

Effect of a PDE9 inhibitor and cGMP on PDE3A activity in vitro

To determine whether the PDE-9 specific inhibitor BAY 73-6691 could also inhibit PDE3A activity, we measured degradation of fluorescently labeled cAMP following co-incubation with PDE3A and increasing concentrations of the PDE9A inhibitor (Figure 2). There were no significant inhibitory effects of BAY 73-6691 on PDE3A activity [0% (1 μM); −1.1%, (10 μM), −5.9% (100 μM), and −7.6% (1 mM)]. These data demonstrate PDE9 does not directly hydrolyze cAMP or inhibit PDE3A activity to any significant extent and therefore should have little effect on meiosis and not contribute to GV retention during *in vitro* oocyte culture experiments. Higher concentrations of the inhibitor were not measured as precipitant forms at doses greater than 1 mM; the slight inhibition observed at the 1 mM concentration may be an artifact.

Increasing concentrations of 8-Br-cGMP were also assayed to determine the dose response for inhibition of PDE3A by cGMP. The lowest concentrations of 1 and 10 μM 8-Br-cGMP produced small, non-significant inhibition of PDE3A activity (−2.8% and −11.8%) (Figure 2) but higher concentrations produced significant (−68.9 (100 μM, −93.4 (1 mM), and −100% (10 mM) ($P < 0.001$). These data verify a dose-dependent inhibitory effect of 8-Br-cGMP on PDE3A hydrolysis of cAMP.

Effect of BAY 73-6691 on mouse oocyte maturation in vitro

Given the limited availability of monkey oocytes for culture experiments, preliminary functional data assessing PDE9 inhibitor activity was obtained using mouse oocytes. Prior to *in vitro* culture experiments with the PDE9 inhibitor, the transcript expression of cGMP-specific PDEs (Pde5A, Pde6A, and Pde9A) in mouse GV oocytes was verified by conventional RT-PCR (data not shown). Only Pde6A and Pde9A were detected and the expression level of Pde9A was twice that of Pde6A. Therefore, mouse GV oocytes were deemed an appropriate model for this preliminary investigation into the effects of the PDE9 inhibitor in mammalian oocytes.

Cumulus-free mouse GV oocytes were cultured in KSOM medium containing 10 μM, 100 μM, or 1 mM BAY 73-6691 for 20–22 hours, and GV retention rates were compared to the non-treated control (no inhibitor). Of the 4 treatment groups, only the oocytes cultured with 1 mM BAY 73-6691 exhibited a modest, but not statistically significant increase in meiotic inhibition (19.0%). No detectable difference was found among the proportion of oocytes cultured in 10 μM (7.4%) and 100 μM (11.8%) BAY 73-6691, compared to the control (7.8%) (Figure 3A).

Effect of 8-Br-cGMP on mouse oocyte maturation in vitro

Cumulus-free GV oocytes were cultured with the membrane permeable cGMP analog, 8-Br-cGMP, to verify the minimum concentration of exogenous cGMP required to impede spontaneous meiosis *in vitro* (Figure 3B). We observed that meiosis was inhibited in a typical dose-response fashion as indicated by the retention of the germinal vesicle. Compared to the non-treated control (7.8% GV intact), 500 μM, 1 mM, and 10 mM 8-Br-cGMP maintained GV arrest in a significantly higher proportion of the total oocytes cultured [21.1%, 57.8%, and 97.8%, respectively]. There was no difference in the proportion of oocytes that maintained meiosis arrest in 100 μM 8-Br-cGMP (8.8%) when compared to the control treatment.

Inhibitory effects of 8-Br-cGMP and BAY 73-6691 co-treatment on spontaneous meiosis resumption in vitro

Cumulus-free mouse GV oocytes were co-cultured with 8-Br-cGMP and BAY 73-6691 in a multiple combinatorial approach with increasing concentrations of each compound. The proportion of oocytes that remained in the GV stage were graphically reported in Figure 3B. At every level of 8-Br-cGMP tested, the addition of BAY 73-6691 induced significant increases in the proportion of oocytes which retained the germinal vesicle throughout the culture period. As the level of 8-Br-cGMP increased, so did the impact of the PDE9 inhibitor, allowing fewer oocytes to exit prophase I arrest. The inclusion of 0 μM, 10 μM, 100 μM, and 1 mM BAY 73-6691 to the 8-Br-cGMP supplemented culture media, substantially increased the proportion of oocytes maintaining GV arrest (non-supplemented control = 11.8%); 8-Br-cGMP 100 μM [8.8%, 11.4%, 18.8%, and 28%], 500 μM [21.1%, 38.1%, 74.5%, and 66.5%], and 1 mM [57.8%, 74.5%, 93.9%, and 94.0%] respectively (Figure 3B).

Discussion

The regulatory roles of cyclic nucleotides and selected PDEs in the maturing follicle are well established (19). Elevated levels of cAMP within the GV oocyte maintain the meiotic arrest state by supporting PKA activity, thus preventing further downstream signaling in the meiosis activation pathway (31). PDEs target specific cyclic nucleotides during meiotic resumption and PDE3A has been identified as the dominant enzyme that actively hydrolyzes cAMP to promote oocyte maturation (6, 32). Signaling molecules in the oocyte, upstream of PDE3A, work in concert to prevent premature initiation of meiosis by inhibiting PDE3A activity. In particular, cGMP is produced in the cumulus granulosa cells and diffuses into the oocyte through gap junctions. High levels of cGMP in the oocyte inhibits PDE3A hydrolytic activity so that elevated levels of cAMP are sustained and the oocyte remains in meiotic arrest, allowing time for the accumulation of cytoplasmic competence (33). Following LH-surge induced closure of the gap junctions, the meiosis inhibiting cGMP is no longer supplied to the oocyte, but residual quantities must be reduced before PDE3A activity can begin degrading cAMP (34, 35).

Until now, it was unclear which PDE enzyme is present in the fully grown GV oocyte to participate in hydrolyzing the residual cGMP following gap-junction closure. We identified PDE9A as the only cGMP-specific degrading gene transcribed in the primate GV oocyte prior to LH-induced initiation of meiosis (Figure 1A), and a potential candidate responsible for removal of inhibitory cGMP from the oocyte. Microarray data collected for transcriptome analysis of human metaphase II oocytes confirms that of the three cGMP-specific PDEs, *PDE9A* is greatly expressed above background control values while *PDE5A* and *PDE6A* barely register (36). Additional studies on kinetics analysis calculate PDE9A as having a K_m of 170 nM for cGMP making it the PDE with the greatest affinity for cGMP (37). Taken together, it is possible that PDE9A may be the dominant (if not only) cGMP-specific degrading enzyme in the oocyte; however future experiments are required to directly measure PDE9A activity in peri-ovulatory primate oocytes.

Our experiments also support a functional role of PDE9A during meiosis regulation. First, we demonstrated using a fluorescence polarization assay that the membrane permeable cGMP analog 8-Br-cGMP inhibits PDE3A activity in a dose dependent fashion, but the PDE 9 inhibitor BAY 73-669 does not. (Figure 2). While a slight decrease of PDE3 was observed at 1 mM with the PDE9 inhibitor, minor precipitation of the compound was observed and this could have interfered with the ability of the detector to precisely measure fluorescence affecting the reliability the assay at this level. Although the concentrations of 8-Br-cGMP in our experiments needed to inhibit PDE3 were very high, a dose response was observed and no precipitation of the compound was noted. Using [^{3}H] cGMP, Vaccari, et al., demonstrated that levels of cGMP in the nanomolar range (IC_{50} = 96.9 nM) inhibited PDE3 derived from mouse oocytes extracts in a typical dose response fashion (18). The concentration of the cGMP isozyme, 8-Br-cGMP, needed in our experiments to completely inhibit PDE3A activity was much higher and non-physiologic, this is most likely due to the imidizole ring substitution, required to make the compound soluble and membrane permeable; this increases the IC_{50} 65-fold compared to unmodified cGMP (38). Although a higher dose is required to exert a similar effect as native cGMP, 8-Br-cGMP is widely used in oocyte meiosis studies and is a suitable model agent for *in vitro* experiments (39, 40).

In our mouse oocyte culture, 8-Br-cGMP, prevented spontaneous *in vitro* maturation in up to nearly 100% of the oocytes at 10 mM, with a dose response similar to that reported in hamster, mouse, and pig oocytes (41–43). The PDE 9 inhibitor BAY 73-6691 alone had no inhibitory effect on spontaneous resumption of meiosis, suggesting that the cumulus-free oocyte is not a significant source of cGMP so that endogenous levels are low without the

cumulus cell contribution. In addition, the rate of cGMP degradation in GV oocytes is unknown. Lower concentrations of the PDE9 inhibitor were not sufficient to stop the theoretically rapid hydrolysis of cGMP and may explain why the highest concentration (1 mM) had only a minor, and non-statistically significant inhibitory effect. It is possible that a higher concentration of BAY 73-6691 may be required to adequately slow the hydrolysis of cGMP in order to observe an impact on meiotic resumption. However, BAY 73-6691 and 8-Br-cGMP combined in culture showed a synergistic effect where PDE9 inhibition augmented the potency of cGMP to sustain meiotic arrest (Figure 3B). Addition of 100 μM or 1 mM BAY 73-6691 to 1 mM of 8-Br-cGMP was enough to prevent germinal vesicle breakdown in 94% of the oocytes which is similar to the percentage of inhibited oocytes cultured in 10 mM 8-Br-cGMP alone. In short, by inhibiting PDE9 activity in the oocyte, the amount of cGMP required to suppress PDE3A activity was decreased 10-fold. From these preliminary data we can conclude that PDE9A participates in the oocyte maturation pathway upstream of PDE3A and cGMP (Figure 4).

Previous work in our laboratory evaluated the pharmacological PDE3 inhibitor, ORG 9935, for its ability to prevent spontaneous and gonadotropin-induced oocyte maturation and as a contraceptive agent for socially-housed harems of rhesus monkeys (14–16, 26). ORG 9935 proved to be an effective inhibitor of meiosis both *in vitro* and in vivo and prevented pregnancy when present at serum levels above 300 nMol/L, but problems associated with drug bioavailability and tolerability prevented further investigation of this compound.

These experiments suggest that blocking PDE9 activity may be useful as part of a contraceptive strategy. Recent data demonstrated that when the catalytic domain of PDE9A was injected into mouse follicle enclosed oocytes, cAMP was drastically reduced after 1 hour and nuclear maturation resumed after 2 hours (19). The decrease in cAMP was not a direct result of PDE9A hydrolytic activity but rather as a result of decreased cGMP in the oocyte via PDE9A degradation. Although an enzymatic extract was used for these data, the ability of PDE9A to effectively reduce cGMP in oocyte cytoplasm appears to be robust (19). By extension, targeted inhibition of PDE9A would prevent cGMP breakdown and prolong the time to nuclear maturation, following the LH surge, through cGMP inhibition of PDE3A.

We theorize that a low-dose combination of PDE inhibitors targeting specific enzymes localized to the immature oocyte and granulosa cells may represent a novel, non-hormonal based contraceptive strategy. For example, tolerability to PDE3A blockade could be improved through dose reduction supported by the addition of a PDE9A inhibitor. In a theoretical model system, a PDE5 inhibitor (several currently approved drugs are available (44)) could also be included to inhibit cGMP degradation in the granulosa cells so that a higher concentration of cGMP would be available to diffuse through the gap junctions into the oocyte. Similarly, the cAMP hydrolyzing PDE4 is the only isoform demonstrated thus far to be active in human granulosa cells, and an inhibitor to PDE4 would also potentially supply the oocyte with greater levels of inhibitory cAMP (45, 46). Following the gap junction closure event, degradation of cGMP in the oocyte would be blocked by the PDE9 inhibitor which in turn would potentiate the action of the PDE3A inhibitor, preventing hydrolysis of cAMP. This synergistic approach to arrest of oocyte maturation with a low-dose selective “cocktail” approach to phosphodiesterase inhibition in the periovulatory follicle may reduce the risk of off-target side effects in a novel nonhormonal strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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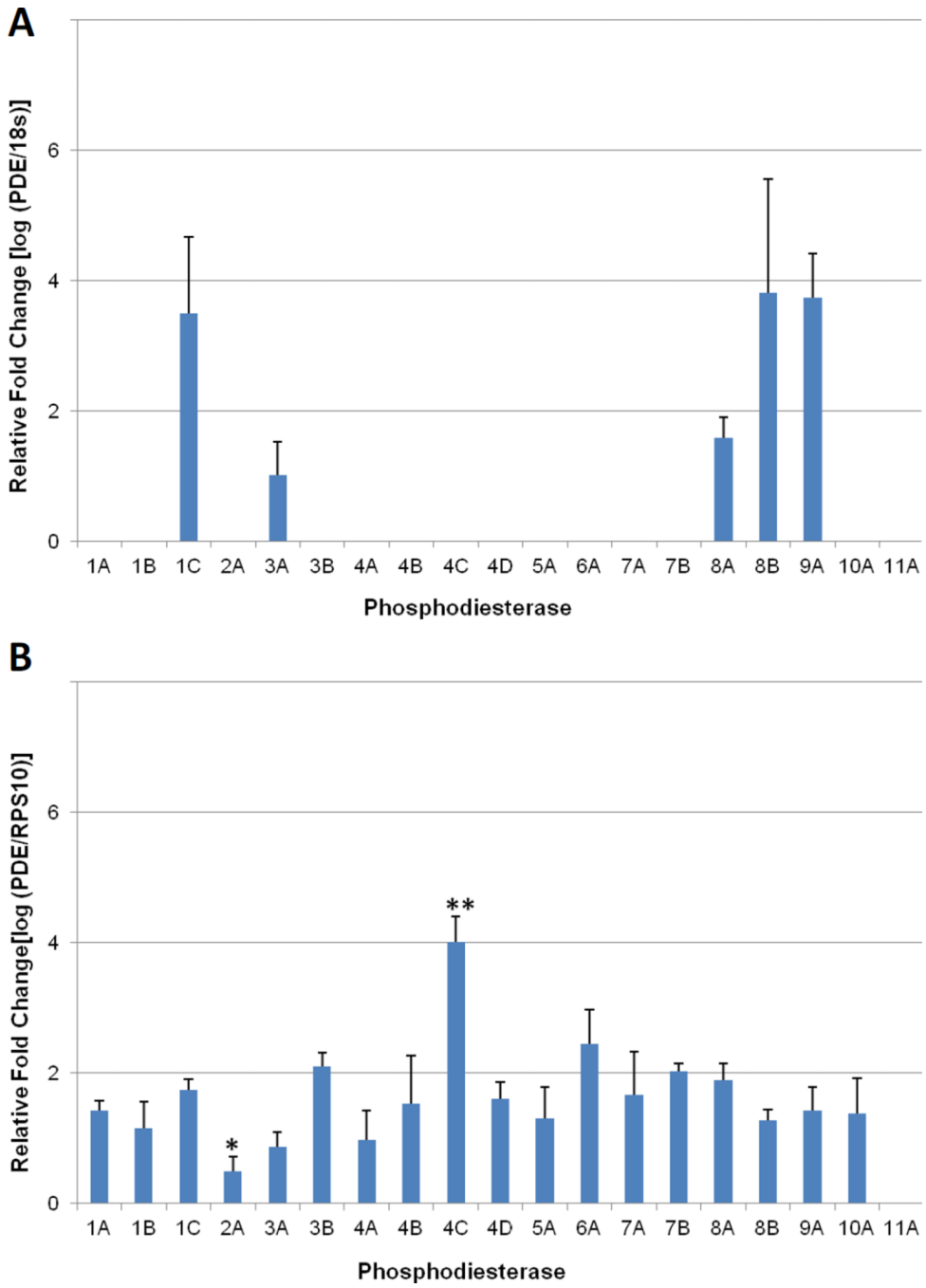

Figure 1.

Quantitative PCR (qPCR) profiling of PDE expression in rhesus monkey GV oocytes and granulosa cells collected from pre-ovulatory antral follicles. (A) Of the 19 PDE genes that were assayed, only *PDE1C, PDE3A, PDE8A, PDE8B*, and *PDE9A* were detectable in the GV oocyte. (B) PDE expression in granulosa cells, *PDE11A* was the only isoform not detectable in any of the samples evaluated. Expression ratios are based on a comparison to the endogenous control gene; *18s-rRNA* for oocytes and *RPS10* for granulosa cells. Error bars indicate standard deviation. One Way ANOVA was performed with a Student-Newman-Keuls post hoc test to determine significant changes in expression with $P < 0.001$.

* Indicates expression is significantly less than all other isoforms. ** Expression is significantly greater than all other isoforms.

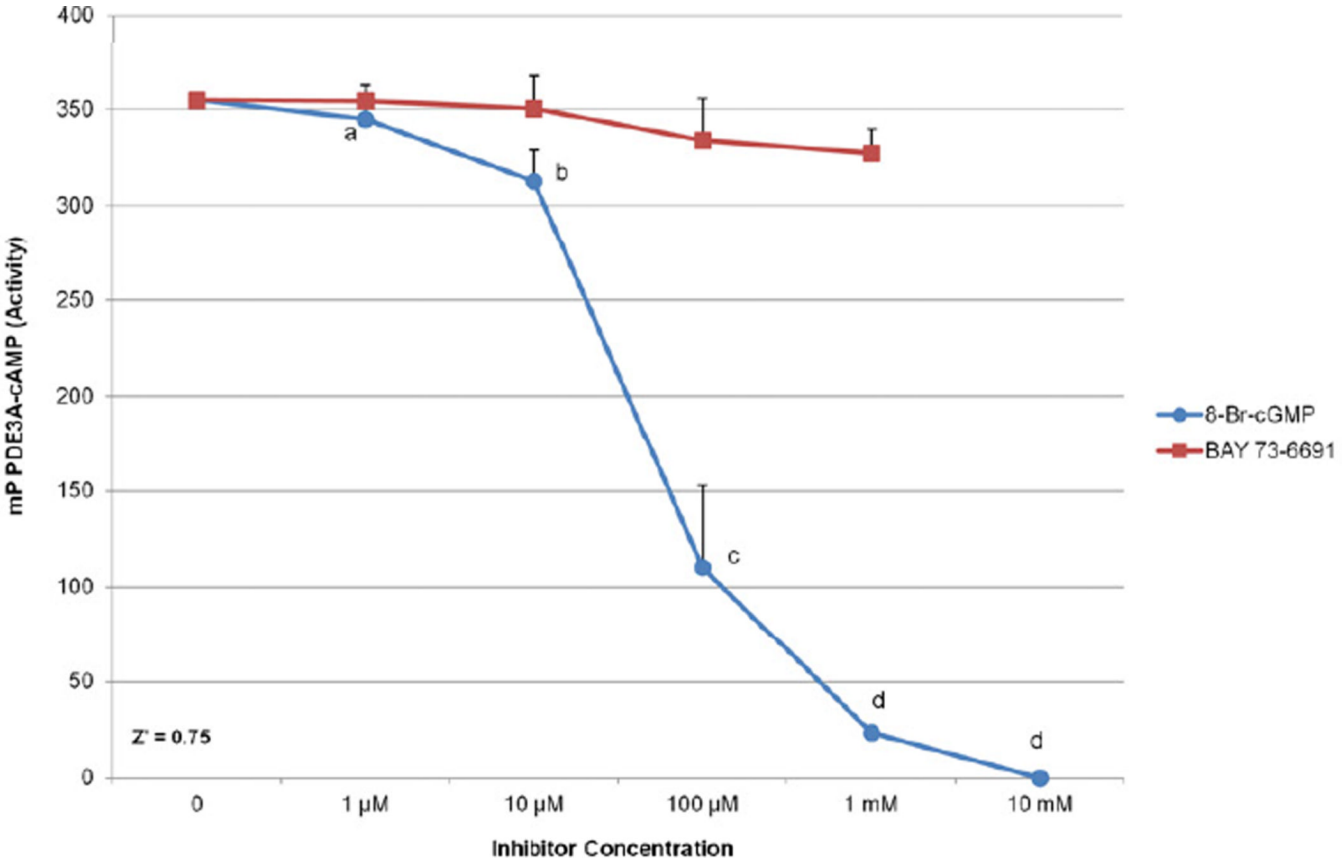

Figure 2.

Florescence polarization assay for PDE3A activity. Increasing concentrations of the PDE9 inhibitor, BAY 73-6691, were evaluated for inhibitory properties against PDE3A activity at 0 µM (no inhibitor), 1 µM, 10 µM, 100 µM, and 1 mM (Red). Similarly, the cGMP analog, 8-Br-cGMP, was assayed at 0 µM (no inhibitor), 1 µM, 10 µM, 100 µM, 1 mM and 10 mM (Blue). No significant changes were observed for the PDE9 inhibitor. Different letters indicate a significant decrease in PDE3A and Z' = 0.75 for both assays. Significant changes in activity were analyzed by ANOVA followed by posthoc comparison with Student-Newman-Keuls test with $P<0.001$. Error bars indicate the first standard deviation.

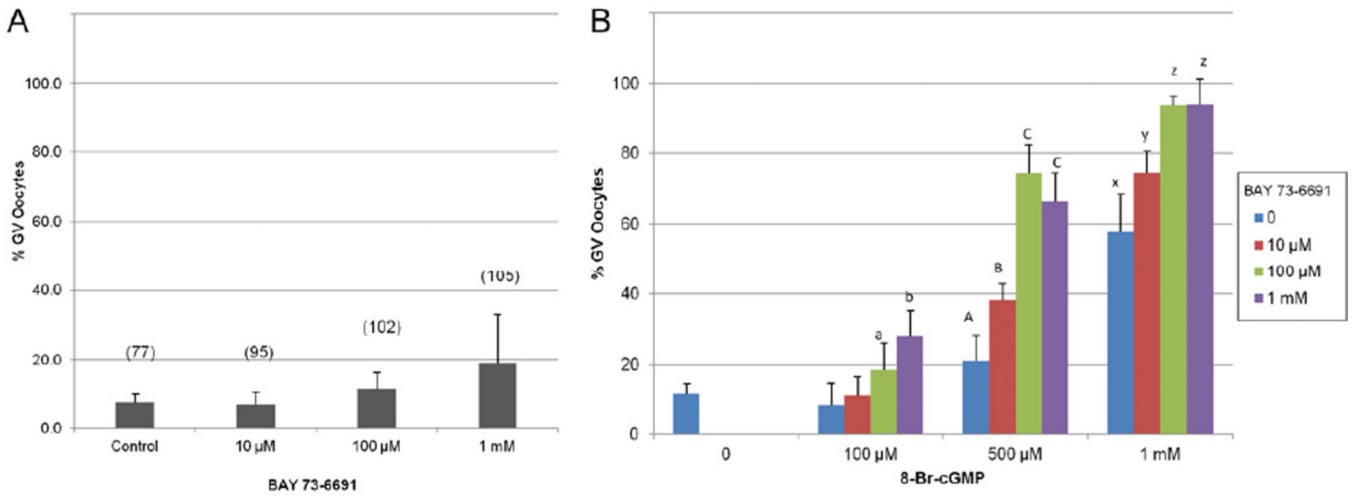

Figure 3.

Percentage of mouse oocytes that retained the GV after 20–22 hours of culture with (A) the selective PDE9 inhibitor BAY 73-6691, or (B) a combination of both 8-Br-cGMP and BAY 73-6691. Error bars represent the standard deviation; value in parenthesis represents the total number of oocytes in each treatment group for (A). Different letters at each level of 8-Br-cGMP in (B) represent a significant change from the control and determine by Chi Square analysis with $P<0.001$.

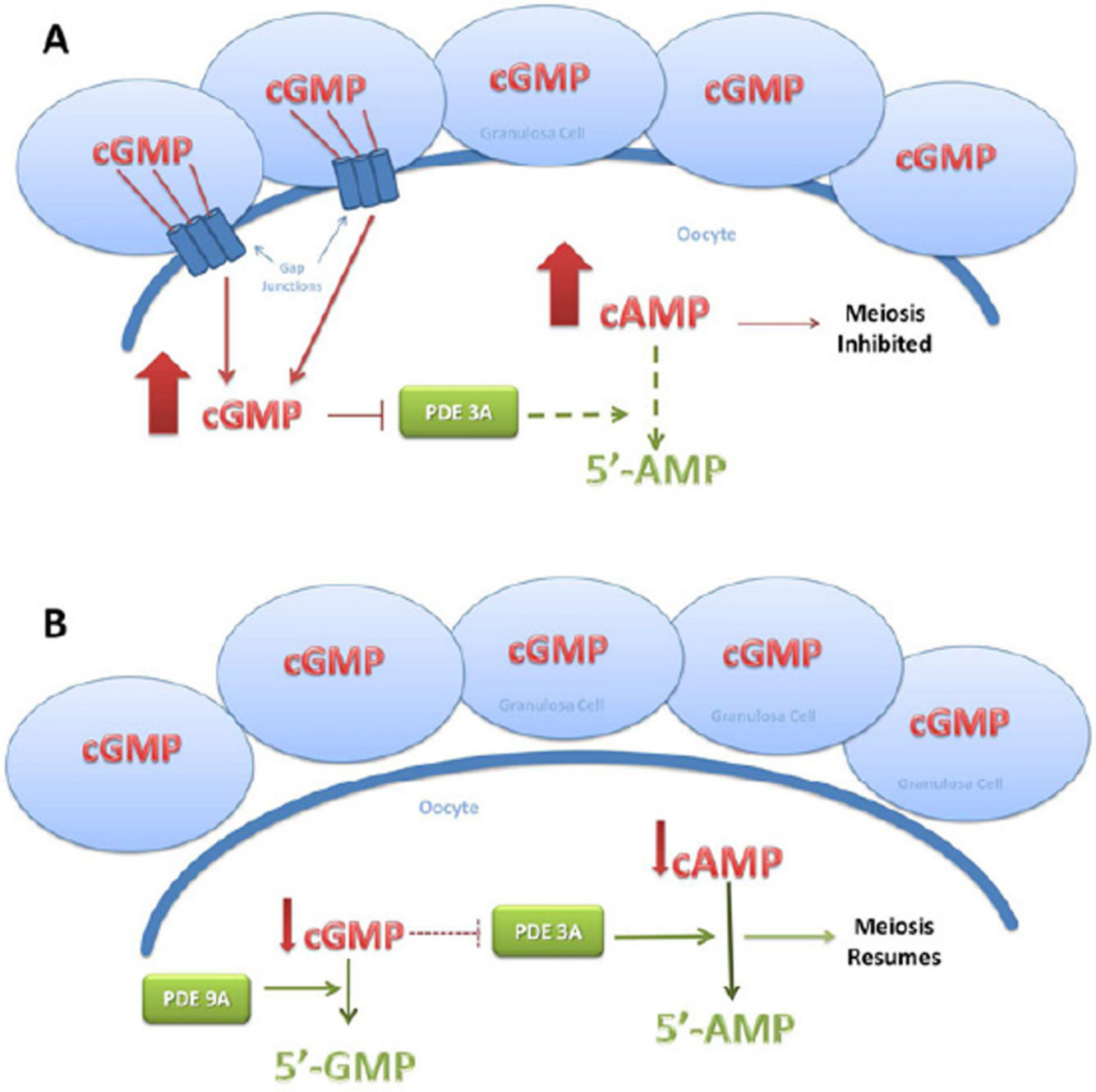

Figure 4.

Proposed PDE9 activity pathway in the oocyte. (A) cGMP from granulosa cells is actively transported through gap junctions into the GV oocyte. Elevated levels of cytoplasmic cGMP inhibit PDE3A hydrolysis of cAMP and maintain the oocyte in meiotic arrest. Based on our data, following the LH surge, gap junctions are closed, and residual cGMP is degraded by PDE9 (B). Decreased levels of cGMP permit PDE3A mediated hydrolysis of cAMP, lowering the cytoplasmic concentration and inducing meiotic resumption.