Multiple cAMP phosphodiesterases act together to prevent premature oocyte meiosis and ovulation

Giulia Vigone, Leia C. Shuhaibar, Jeremy R. Egbert, Tracy F. Uliasz, Matthew A. Movsesian, and Laurinda A. Jaffe

Endocrinology
Endocrine Society

Submitted: January 03, 2018
Accepted: March 22, 2018
First Online: March 28, 2018

Endocrinology; Copyright 2018 DOI: 10.1210/en.2018-00017

PDE activity maintains ovarian follicle arrest

Multiple cAMP phosphodiesterases act together to prevent premature oocyte meiosis and ovulation

Giulia Vigone[1]*, Leia C. Shuhaibar[1], Jeremy R. Egbert[1], Tracy F. Uliasz[1], Matthew A. Movsesian[2], and Laurinda A. Jaffe[1]*

[1]*Department of Cell Biology, University of Connecticut Health Center, Farmington, CT, USA*

[2]*Cardiology Section, VA Salt Lake City Health Care System and Division of Cardiovascular Medicine, University of Utah School of Medicine, Salt Lake City, UT, USA*

Received 03 January 2018. Accepted 22 March 2018.

Luteinizing hormone (LH) acts on the granulosa cells that surround the oocyte in mammalian preovulatory follicles to cause meiotic resumption and ovulation. Both of these responses are mediated primarily by an increase in cAMP in the granulosa cells, and activity of the cAMP phosphodiesterase PDE4 contributes to preventing premature responses. However, 2 other cAMP-specific phosphodiesterases, PDE7 and PDE8, are also expressed at high levels in the granulosa cells, raising the question of whether these phosphodiesterases also contribute to preventing uncontrolled activation of meiotic resumption and ovulation. Using selective inhibitors, we show that inhibition of PDE7 or PDE8 alone has no effect on the cAMP content of follicles, and inhibition of PDE4 alone has only a small and variable effect. In contrast, a mixture of the 3 inhibitors elevates cAMP to a level comparable to that seen with LH. Correspondingly, inhibition of PDE7 or PDE8 alone has no effect on meiotic resumption or ovulation, and inhibition of PDE4 alone has only a partial and slow effect. However, the fraction of oocytes resuming meiosis and undergoing ovulation is increased when PDE4, PDE7, and PDE8 are simultaneously inhibited. PDE4, PDE7, and PDE8 also function together to suppress the premature synthesis of progesterone and progesterone receptors, which are required for ovulation. Our results indicate that 3 cAMP phosphodiesterases act in concert to suppress premature responses in preovulatory follicles.

Multiple cAMP phosphodiesterases function together to maintain the mouse ovarian follicle in an arrested state prior to stimulation by luteinizing hormone.

Introduction

In cells in which physiological responses are elicited by hormones that elevate cAMP, it is critical that cAMP stays low prior to hormonal stimulation. To ensure that cAMP-dependent processes do not occur prematurely, cAMP phosphodiesterases are continuously active in many tissues (see Discussion), including granulosa cells of the ovarian follicle (1-3). The mammalian ovarian follicle is a spherical complex of thousands of granulosa cells surrounding an oocyte in the center. The oocyte's meiotic cell cycle is paused at prophase, and cyclic nucleotide levels must be precisely controlled to maintain the arrest until with each reproductive cycle, a subset of follicles respond to luteinizing hormone (LH) (4-6). LH acts on its receptors in the granulosa cells to activate the G-protein G_s, thus activating adenylyl cyclase and elevating cAMP, and the cAMP rise in the granulosa cells is a primary stimulus for reinitiation of meiosis in the oocyte and for ovulation (5, 6). LH signaling triggers other responses as well, including activation of the GLYT1 glycine transporter in the oocyte, allowing cell volume regulation (7) and the activation of genes leading to granulosa cell luteinization (8). All of these responses occur following a

preovulatory increase in LH, during which serum levels of LH stay elevated for a period of several hours (9). Although the LH receptor activates other G-proteins in addition to G_s (5), prevention of untimely cAMP elevation is essential for preventing premature responses in the follicle.

Previous studies have indicated that activity of the cAMP phosphodiesterase PDE4 contributes to preventing meiosis and ovulation from occuring prematurely. PDE4 inhibitors promote resumption of meiosis in isolated follicles (2, 3), and injection of some (but not other) PDE4 inhibitors into rats causes progesterone production and ovulation (10). However, PDE4 accounts for only 20-30% of the total cAMP phosphodiesterase activity in mouse preovulatory ovaries (11, 12), and for only 15-20% of the cAMP phosphodiesterase activity in bovine and rat mural granulosa cells (13, 14).

In preovulatory mural granulosa cells of mice, rats, and humans, levels of mRNA encoding the cAMP-specific phosphodiesterases PDE7A, PDE7B, PDE8A, and PDE8B are much higher than those encoding the 4 isoforms of PDE4 (14-16). Furthermore, PDE8 accounts for ~40% of the cAMP phosphodiesterase activity in bovine and porcine mural granulosa cells (13, 17). Studies of a T-cell line have indicated that inhibition of PDE7 and PDE8 results in phosphorylation of a set of proteins distinct from those that are phosphorylated when PDE4 is inhibited (18). These findings, as well as extensive evidence that PDE4, PDE7, and PDE8 often act together in other cells to control cAMP-dependent processes (see Discussion), led us to investigate the function of these 3 phosphodiesterases in mouse ovarian follicles. Here we use a new PDE7 inhibitor, alone or in combination with PDE4 and PDE8 inhibitors, to test the effects of inhibiting these phosphodiesterases on cAMP levels, meiotic resumption, ovulation, and production of progesterone and its receptors.

Materials and Methods

Phosphodiesterase inhibitors

The PDE4 inhibitor rolipram was obtained from Tocris BioScience (Bristol, UK). The PDE7 and PDE8 inhibitors, referred to here as PDE7i and PDE8i (also called PF-04957325), were obtained from Pfizer Worldwide Research and Development (Groton, CT). The chemical structure of the PDE7 inhibitor is shown in Fig. S1. For comparison, this figure also shows the previously published structures of rolipram (19) and PF-04957325 (20). PF-04957325 is now available from MedChemExpress (Monmouth Junction, NJ). Stock solutions were made in DMSO and stored at -80°C. Final DMSO concentrations after dilution were as follows: 1 μM PDE7i, 0.003% DMSO; 1 μM PDE8i, 0.002% DMSO; 5 μM rolipram, 0.005% DMSO; 20 μM rolipram, 0.02% DMSO; 5 μM rolipram + 1 μM PDE7i + 1 μM , 0.01% DMSO. By itself, 0.1% DMSO did not cause meiotic resumption or ovulation in cultured ovarian follicles, and did not affect the time course of meiotic resumption in response to LH (Fig. S2). Since the highest concentration of DMSO that was used in the solutions of phosphodiesterase inhibitors was less than this (0.02%), DMSO was not included in controls without phosphodiesterase inhibitors.

Rolipram has an IC_{50} for PDE4 of 70-140 nM, measured with 1 μM cAMP substrate, while IC_{50} values for all other cyclic nucleotide phosphodiesterases are >100 μM (21-24). The PDE7 and PDE8 inhibitors are also highly selective. K_i values for the PDE7 and PDE8 inhibitors were determined as previously described (25), for a member of each cyclic nucleotide phosphodiesterase family. These data, shown in Table 1, were supplied by Steve Jenkinson (Pfizer).

In brief, PDE activity was measured using a scintillation proximity assay (SPA) that measures the inhibition by the indicated compounds of the activity of the human recombinant phosphodiesterases listed in Table 1 (except for PDE6, which was extracted from bovine retina) as determined *in vitro* using lysates or FLAG-tag purified enzymes. The assays were performed in a 384-well format. These assays were used to measure the hydrolysis of [^{3}H]cGMP to the 5' nucleotide [^{3}H]GMP, or of [^{3}H]cAMP to the 5' nucleotide [^{3}H]AMP, as indicated in Table 1. The enzyme stocks were thawed slowly and diluted in assay buffer containing 50 mM Tris HCl buffer (pH 7.5 at room temperature) and 1.3 mM $MgCl_2$. Additionally, the PDE1 assay buffers contained 2.8 mM $CaCl_2$, and the activator calmodulin at a final assay concentration of 100 units/ml. The enzyme concentration for each isoform was determined by enzyme titration experiments to achieve 20-30% substrate turn-over at 30 minutes, with the exception of the PDE6 reactions, which were incubated for 60 minutes. The final substrate concentration used was at sub-K_m levels (20 nM for [^{3}H]cAMP and 50 nM for [^{3}H]cGMP) so that IC_{50} values would approximate the K_i values. In all cases, these reactions were well within the linear portion of the assay.

The reaction was stopped by the addition of phosphodiesterase SPA beads (Perkin Elmer) at a final assay concentration of 0.2 mg/well. The PDE9 assay required the extra addition of a potent PDE9 inhibitor, PF-00509783, at a high concentration (10 µM) prior to adding beads in order to completely stop the reaction. The products, [^{3}H]GMP and [^{3}H]AMP, bound preferentially to yttrium silicate SPA beads and were detected by scintillation counting in a Microbeta Trilux Counter (PerkinElmer). The inhibition of enzyme activity was calculated relative to the activity of uninhibited controls (DMSO) and fully inhibited (high concentration of known inhibitor). To determine IC_{50} values, the compounds were tested in duplicate using a half-log dilution scheme with the top concentration of 10 µM. The corresponding IC_{50} values of the compounds for the inhibition of PDE activities were determined from the concentration-effect curves by using a four-parameter logistic fit. Apparent K_i values were estimated by using the Cheng-Prusoff equation: - $K_i = (IC_{50})/(1 + [S]/K_m)$, where S is the substrate, and K_m is the substrate concentration at which half the enzyme's active sites are occupied by substrate.

The mean K_i for PDE7i inhibition of PDE7B is 7.8 nM, compared with ≥3.8 µM for all other cyclic nucleotide phosphodiesterase families (Table 1). The K_i for PDE7i inhibition of PDE7A was not determined. The mean K_i for PDE8i inhibition of PDE8B is 0.1 nM, compared with ≥2.5 µM for all other cyclic nucleotide phosphodiesterase families (Table 1). These measurements are close to previously published IC_{50} values for this inhibitor (20, 26, 27). These previous studies reported IC_{50} values for this inhibitor that are ~2-8 times higher for PDE8A than for PDE8B, but still much lower than for all other cyclic nucleotide phosphodiesterase families (see Table 1 legend).

Mice and follicle culture

Prepubertal B6SJLF1 mice (23-25 days old) from The Jackson Laboratory (Bar Harbor, ME) were used for all experiments. Procedures were approved by the animal care committee of the University of Connecticut Health Center.

Ovarian follicles, 320-400 µm in diameter, were isolated using fine forceps. Before use, the follicles were cultured for 24-30 hours on Millicell organotypic membranes (Merck Millipore Ltd, Cork, IRL; PICMORG50) (28, 29). 3 mg/ml BSA was included in the culture medium in place of serum. 1 nM (30 ng/ml) of follicle stimulating hormone (FSH) was included in the medium to stimulate follicle growth and expression of LH receptors. The use of FSH at 1 nM instead of 0.3 nM (10 ng/ml) that we have used previously was an improvement; with 1 nM

FSH, 100% of follicle-enclosed oocytes underwent nuclear envelope breakdown by 6 hours after LH exposure, whereas with 0.3 nM FSH, the percentage was typically ~80% (30, 31). LH was used at 0.1, 1, 10, or 300 nM (0.3 ng/ml - 10 μg/ml), as indicated. Highly purified ovine FSH (AFP7558C) and ovine LH (oLH-26) were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA).

Observations of follicles on Millicell membranes were made using an upright microscope with a 20x/0.4 NA long-working distance objective. LH or phosphodiesterase inhibitors were applied to the medium in the dish holding the Millicell membrane, and oocytes within follicles were observed for the presence or absence of a nuclear envelope and nucleolus at 1-hour intervals. Photographs of follicles were taken on the Millicell membranes. Ovulation was scored at 24 hours.

cAMP measurement

Follicles were sonicated in 0.1 M HCl, and cAMP was measured with a direct cAMP ELISA kit from Enzo (Farmingdale, NY) (ADI-900-066). This kit is highly selective for cAMP, and shows only 0.33% cross reactivity with AMP, 0.12% with ATP, and <0.001% with cGMP, GMP, GTP, cUMP, and CTP (manufacturer's product information sheet). Inter-assay precision was determined by comparing the standard curves obtained in 16 separate assays, using cAMP solutions prepared from a stock solution provided with the kit. The coefficient of variation (standard deviation/mean x 100) at the mid-point concentration was 14.5%. Intra-assay precision was determined by assaying 14 replicates of the midpoint concentration in a single assay; the coefficient of variation was 1.2%.

Progesterone measurement

Progesterone in the culture media was measured using an ELISA kit from Cayman Chemical Company (Ann Arbor, MI) (582601). This kit is selective for progesterone, but shows a small amount of cross-reactivity with prenenolone (14%), 17β-estradiol (7.2%), 5β-pregnan-3α-ol-20-one (6.7%), and 17α-hydroxyprogesterone (3.6%) (manufacturer's product information sheet). Inter-assay precision was determined by comparing the standard curves obtained in 16 separate assays, using progesterone solutions prepared from a stock solution provided with the kit. The coefficient of variation at the mid-point concentration was 24.3%. Intra-assay precision was determined by assaying 7 replicates of the midpoint concentration in a single assay; the coefficient of variation was 6.2%.

Western blotting

Samples for western blotting were prepared by sonicating follicles in Laemmli sample buffer containing 75 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, 1 mM Pefabloc (Sigma-Aldrich, St. Louis, MO), and Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The progesterone receptor (PGR) antibody was obtained from Abcam (ab 16661, rabbit monoclonal, clone SP2; Table 2). This antibody recognizes both PGR-A and PGR-B isoforms (32). The tissue culture supernatant was used at a dilution of 1:500. The PGR antibody was detected using a fluorescent secondary antibody (IRDye800 CW, 926-32211 from LICOR BioSciences, Lincoln, NE). Binding of the secondary antibody was quantified using the LICOR BioSciences Odessey infrared imaging system. Variability in the amount of total protein loaded in each lane was <5% as confirmed by densitometry of staining with Swift Membrane Stain (G-Biosciences, St. Louis, MO).

Measurement of cAMP phosphodiesterase activity in follicle lysates

Methods were the same as previously described (16), except that ^{3}H-cAMP was used instead of ^{3}H-cGMP.

Statistics

Data were analyzed by one-way ANOVA or unpaired t-test followed by the Holm-Sidak correction for multiple comparisons, as appropriate, using Prism software (GraphPad, La Jolla, CA). Percent data were transformed by arc sine square root transformation prior to analysis. All values indicate mean ± s.e.m. Numbers in parentheses indicate the numbers of independent experiments, and asterisks and different letters indicate significant differences ($p<0.05$). Each experiment included 8-16 follicles.

Results

PDE4, PDE7, and PDE8 act together to suppress premature cAMP elevation in preovulatory follicles

The LH receptor stimulates cAMP formation by activating the G protein G_s in granulosa cells, and the resulting elevation of cAMP is a major component of the signal that causes meiotic resumption from prophase arrest (5, 6). As background for investigating the role of phosphodiesterases in suppressing premature cAMP elevation, we first characterized the LH-induced cAMP increase under our experimental conditions, by measuring the cAMP content of mouse preovulatory follicles at various times after application of various concentrations of LH (Fig. 1A). Concentrations of LH that were sufficient to maximally stimulate meiotic resumption in our culture system (≥10 nM) (see Fig. 2B below) increased the cAMP content by ~5-fold at 30 minutes. The cAMP content then declined gradually, but was still above baseline at 4 hours (Fig. 1A). These cAMP levels are similar to those determined in a previous study in which the LH receptor of mouse ovarian follicles was stimulated with human chorionic gonadotropin (33).

A smaller and slower elevation of cAMP occurred with 1 nM LH (Fig. 1A), corresponding to a slower resumption of meiosis (Fig. 2B). At 0.5 to 3 hours after 1 nM LH addition, cAMP content of the follicles was not statistically different from that of follicles that were incubated without LH (Fig. 1A). However, measurements at 4 hours after addition of 1 nM LH showed a significant increase in cAMP (Fig. 1A). 1 nM is similar to the peak serum concentration of LH during the preovulatory surge in mice (9, 34), although the concentration dependence of meiotic resumption in response to purified ovine LH applied to follicles in vitro may not be identical to that when the native hormone is delivered to follicles in vivo.

To investigate which cAMP phosphodiesterases contribute to suppressing premature elevation of cAMP prior to LH exposure, we measured the cAMP content of follicles after exposure to selective inhibitors of PDE4 (rolipram, 5 μM), PDE7 (1 μM), or PDE8 (1 μM), alone or in combination. The inhibitor concentrations were chosen based on the in vitro selectivity data described in Table 1. Because inhibitor concentrations in the cytoplasm may be lower than those in the medium, due to limited permeability of the plasma membrane, we used the highest concentrations at which the inhibitors showed selectivity among different phosphodiesterases. At 5 μM, rolipram inhibition of PDE7 and PDE8 is insignificant, since the IC_{50} for PDE7 and PDE8 is >100 μM (21-24). At 1 μM, there could be minor inhibition by the PDE7 inhibitor of PDE4 and PDE8, and minor inhibition by the PDE8 inhibitor of PDE4 and PDE7, based on measurements of the effects of these inhibitors on activity of recombinant proteins in vitro (Table 1). Higher concentrations of the PDE7 and PDE8 inhibitors were not tested because of the possibility of cross-reactivity with other phosphodiesterases, and these inhibitors were used at 1 μM for all subsequent experiments.

Measurements made at 30 or 60 minutes after application of a mixture of the 3 inhibitors showed little or no increase in cAMP content (Fig. 1A), consistent with the relatively slow action of these inhibitors on biological responses as described below. However, when measurements were made at 2 or 4 hours, the 3 inhibitors together increased the cAMP content by 2-4 fold (Fig. 1A,B).

A 4-hour treatment with rolipram alone caused a small increase in cAMP content in 2 of 3 experiments, but the mean value for the 3 experiments was not statistically different from the cAMP content with no treatment. A 4-hour treatment with either the PDE7 inhibitor or the PDE8 inhibitor had no detectable effect (Fig. 1B). A large increase in cAMP content was seen, however, in response to a 4-hour treatment with a mixture of the 3 inhibitors (Fig. 1B), indicating that these 3 phosphodiesterases act together to maintain cAMP at a low level prior to LH exposure. This synergistic action of the 3 cAMP inhibitors when applied together was also seen in various biological responses as described below.

PDE4, PDE7, and PDE8 act together to suppress premature meiotic resumption in follicle-enclosed oocytes

To monitor the time course of meiotic resumption, follicles were cultured on the surface of Millicell membranes such that they were slightly flattened, allowing observation of the oocyte and its nucleus within the intact follicle (Fig. 2A) (28). The first obvious sign of meiotic resumption is the breakdown of the nuclear envelope (NEBD), marking the transition from prophase to metaphase. Under the culture conditions used here, 100% of oocytes underwent NEBD by 6 hours after exposure to 10 or 300 nM LH (Fig. 2B). 1 nM LH also caused NEBD in some follicles, but the response was delayed (Fig. 2B), consistent with the lower amplitude and slower time course of cAMP elevation in response to LH (Fig. 1A).

Using this culture system, we investigated the effect of inhibitors of PDE4, PDE7, and PDE8 on maintenance of meiotic arrest. At 6 hours, ~15% of the follicles treated with 5 μM of the PDE4 inhibitor rolipram had undergone NEBD (Fig. 2C). A higher concentration of rolipram, 20 μM, did not increase the fraction of oocytes resuming meiosis (Fig. 2D), consistent with a previous report (2). Therefore, we used 5 μM rolipram for all subsequent experiments, except as noted.

None of the follicles treated with 1 μM of the PDE7 or PDE8 inhibitors had undergone NEBD at 6 hours (Fig. 2C). However, when follicles were treated with a mixture of 5 μM rolipram and 1 μM each of the PDE7 and PDE8 inhibitors, ~60% of the follicles had undergone NEBD at 6 hours (Fig. 2C). These results indicate that PDE4, PDE7, and PDE8 act together to suppress spontaneous NEBD. Mixtures of the PDE4 and PDE7 inhibitors, or of the PDE4 and PDE8 inhibitors, also showed a synergistic stimulation of NEBD (Fig. 2C). Based on their effects on recombinant proteins in vitro (Table 1), 1 μM of the PDE8 or PDE7 inhibitors could cause a slight inhibition of PDE4, if this concentration was reached in the cytoplasm. However, this is unlikely to account for the synergistic effect of combining 1 μM of the PDE7 or PDE8 inhibitors with 5 μM rolipram, since no increase in nuclear envelope breakdown was seen with 20 μM vs 5 μM rolipram (Fig. 2D).

The time course of NEBD in response to the mixture of PDE inhibitors showed an approximately 2 hour delay compared with the response to a saturating level of LH (300 nM) (Fig. 2E). Correspondingly, the cAMP content of follicles was not significantly elevated from baseline until 2 hours after applying the mixture of PDE inhibitors (Fig. 1A). Co-incubation with 0.1 nM or 1 nM LH did not increase the rate of NEBD in response to the mixture of cAMP phosphodiesterase inhibitors (Fig. S3).

When follicles were observed at 24 hours after treatment with rolipram alone, ~70% had undergone NEBD (Fig. 2E). This finding is consistent with previous studies in which follicles were exposed to rolipram for 24 hours (2, 3; David Calebiro, personal communication about the timing of the experiments reported in reference 3). Even at 24 hours, no NEBD was seen in follicles treated with 1 μM of the PDE7 or PDE8 inhibitors alone or in combination (Fig. 2E). However, when these inhibitors were combined with rolipram, the percentage of NEBD at 24 hours increased to 100% vs ~70% with rolipram alone (Fig. 2E).

PDE4, PDE7, and PDE8 act together to suppress premature ovulation

LH causes ovulation at least in part by signaling through G_s to elevate cAMP (5, 33), although signaling through $G_{q/11}$ to elevate IP_3 also contributes (5, 35). At 24 hours after applying LH to isolated follicles in culture, the oocyte has been released, and often a polar body can be seen (Fig. 3A). Some of the granulosa cells are also released. Approximately 80% of follicles treated with 1-300 nM LH ovulated (Fig. 3B). To investigate which cAMP phosphodiesterases contribute to suppressing premature ovulation, we investigated the effects of cAMP PDE inhibitors on this process.

Inhibition of PDE4 alone, using 5 or 20 μM rolipram, caused ovulation in ~20% of follicles (Fig. 3C,D). The PDE7 and PDE8 inhibitors, used at 1 μM, alone or in combination, did not cause ovulation (Fig. 3C). However, when the PDE7 or PDE8 inhibitor, or both, were applied together with rolipram, ~80% of follicles ovulated, as seen with LH (Fig. 3C,E). Thus, as with the stimulation of cAMP elevation and meiotic resumption, PDE4, PDE7, and PDE8 act together to suppress premature ovulation.

PDE4, PDE7, and PDE8 all contribute to suppressing premature production of progesterone and progesterone receptors

An aspect of the LH-induced signaling network that is essential for ovulation, although not for meiotic progression, is the synthesis by the granulosa cells of progesterone and its receptors PGR-A and PGR-B (36-40). Synthesis of progesterone and its receptors started between 1-2 hours after exposure to LH (300 nM) (Fig. 4A,B), consistent with previous studies (32, 38, 41-44).

To determine whether premature progesterone production by the follicle is suppressed by constitutive cAMP phosphodiesterase activity, we measured progesterone released into the medium at 6 hours after application of PDE inhibitors. As for the previous analyses, rolipram was used at a concentration of 5 μM, and PDE7 and PDE8 inhibitors were both used at 1 μM. Application of rolipram alone resulted in a progesterone level ~50% of that seen with LH (Fig. 4C). Application of either the PDE7 or the PDE8 inhibitor alone each resulted in a progesterone level ~20% of that seen with LH (Fig. 4C). A mixture of the 3 inhibitors resulted in a progesterone level that was the same as that seen with LH (Fig. 4C). The effect of the 3 inhibitors on progesterone production was additive, but not synergistic.

To investigate if inhibition of PDE4, PDE7, or PDE8 results in synthesis of progesterone receptors, follicles were treated with inhibitors of each of these phosphodiesterases, alone or in combination (Fig. 4D-F). Treatment for 6 hours with rolipram resulted in a level of PGR protein that was ~40% of that seen with LH, while neither the PDE7 nor the PDE8 inhibitor resulted in detectable PGR protein expression. However, treatment with a mixture of the 3 inhibitors reproduced the rise in PGR protein seen with LH, indicating that these cAMP phosphodiesterases act together to suppress progesterone receptor synthesis.

LH signaling does not cause a rapid decrease in cAMP phosphodiesterase activity

Although LH signaling elevates cAMP by stimulating G_s and increasing the activity of adenylyl cyclase (5), this signaling could be amplified by an LH-induced decrease in cAMP phosphodiesterase activity. To investigate this possibility, we measured cAMP phosphodiesterase activity in lysates of follicles that had been treated with or without LH (300 nM). Because the peak cAMP increase after LH exposure occurs at 30 minutes (Fig. 1A), samples were prepared at this time point. Activity was assayed with 2 different cAMP substrate concentrations, 0.1 μM or 1 μM. No difference in activity was seen as a result of the LH treatment, indicating that at least on the time scale investigated, LH signaling does not decrease cAMP phosphodiesterase activity as detected in cell lysates (Fig. 5). By 2 hours after LH receptor stimulation, the amounts of PDE4D protein and PDE4 activity actually increase (12). These results support the conclusion that although cAMP phosphodiesterases contribute to suppressing spontaneous nuclear envelope breakdown and ovulation prior to the preovulatory increase in LH, the initial rise in cAMP after LH exposure is not amplified by a decrease in cAMP phosphodiesterase activity.

Discussion

Our findings indicate that 3 families of cAMP phosphodiesterases – PDE4, PDE7, and PDE8 – act together in mouse ovarian follicles to suppress premature cAMP elevation, meiotic resumption and ovulation prior to the preovulatory increase in luteinizing hormone. The observation that exposure to PDE7 and PDE8 inhibitors has no effect in the absence of PDE4 inhibition indicates that the amount of PDE4 is sufficient to prevent premature resumption of meiosis under these experimental conditions. PDE4 inhibition alone, however, produces a submaximal response that is potentiated by concurrent inhibition of PDE7 and PDE8. These observations indicate a ‘redundancy’ of phosphodiesterases regulating meiotic arrest and ovulation, with PDE4 by itself being sufficient, but with PDE7 and PDE8 serving as ‘back-up’ regulators.

However, this description of the roles of PDE7 and PDE8 as ‘back-up’ regulators applies only to the specific biological responses that we investigated. cAMP is a pleiotropic second messenger, and there may be unique roles for PDE7 and PDE8, vs PDE4, in particular cAMP-mediated pathways in ovarian follicles. Consistent with this concept, our observations at 24 hours after inhibition of PDE4 activity indicate a more pronounced effect on ovulation than on NEBD.

Studies of other cells also indicate differential effects of inhibition of PDE4, PDE7, and PDE8. Phosphoproteomic analysis of a T-cell line has shown that PDE4 inhibition (when combined with PDE3 inhibition) results in phosphorylation of a set of proteins that is distinct from the set of proteins that are phosphorylated as a result of inhibition of PDE7 and PDE8 (when combined with PDE1 inhibition) (18). Correspondingly, the biological processes regulated by these different phosphodiesterase inhibitor combinations differ (18). Likewise, individual phosphodiesterases have been demonstrated to have distinct functional roles in cardiac myocytes, where raising intracellular cAMP content through PDE2 inhibition has anti-hypertrophic effects in neonatal rat ventricular myocytes, while inhibition of PDE3 generates pro-hypertrophic signals (45). Correspondingly, the subcellular localization of PDE2 differs from that of PDE3 (45). Spatially distinct cAMP phosphodiesterase signaling domains may be present in ovarian follicles as well, where they may contribute to the divergent signaling pathways that control different responses to LH.

Cooperative function of cAMP-hydrolyzing phosphodiesterases has also been found in Leydig cells of the testis, which produce testosterone in response to luteinizing hormone (LH). Inhibition of either of 2 different cAMP-specific phosphodiesterases, PDE4 and PDE8, can induce steroid production in these cells, and inhibition of both of these enzymes increases steroid production more than the sum of either alone (26, 46). However, while PDE4 has the primary role and PDE8 a supporting role in regulating meiotic arrest in granulosa cells, the situation is reversed is in Leydig cells, with PDE8 having the primary role and PDE4 a supporting role.

Multiple cAMP phosphodiesterases act together to suppress cAMP-dependent responses in other cells as well. These include cells of the adrenal cortex (47, 48), smooth muscle (49, 50), adipose tissue (51) and leukocytic cell lines (52, 53). Having more than one type of PDE protein present to suppress premature cAMP elevation could help to ensure that conditions that might reduce the activity of any individual phosphodiesterase would not disrupt essential cellular processes. Other factors, such as cAMP buffering by high concentrations of the regulatory subunits of protein kinase A, may also contribute to preventing premature cAMP-dependent responses (54).

Acknowledgments

We acknowledge the generous contributions of Anne W. Schmidt, Tom Chappie, Patrick Verhoest, Steve Jenkinson, and Christopher J. Schmidt (Pfizer Worldwide Research and Development), who supplied the PDE7 and PDE8 inhibitors, as well as data on their selectivity. We also thank Joe Beavo, Dan Bernard, Stefan Brocke, Paul Epstein, and Frank Menniti for helpful discussions, and Judith Krall for technical assistance.

This work was supported by a grant from the Eunice Kennedy Shriver National Institute of Child Health and Human Development to LAJ (R37HD014939).

Eunice Kennedy Shriver National Institute of Child Health and Human Development http://dx.doi.org/10.13039/100009633, R37HD014939, Laurinda A. Jaffe

*Correspondence: Giulia Vigone or Laurinda A. Jaffe, Department of Cell Biology, University of Connecticut Health Center, Farmington, CT 06030, USA. Email: vigone@uchc.edu, or ljaffe@uchc.edu.

DISCLOSURE STATEMENT:
The authors have nothing to disclose.

References

1. Conti M, Kasson BG, Hsueh AJ. Hormonal regulation of 3',5'-adenosine monophosphate phosphodiesterases in cultured rat granulosa cells. Endocrinology 1984; 114:2361-2368

2. Tsafriri A, Chun SY, Zhang R, Hsueh AJ, Conti M. Oocyte maturation involves compartmentalization and opposing changes of cAMP levels in follicular somatic and germ cells: studies using selective phosphodiesterase inhibitors. Dev Biol 1996; 178:393-402

3. Lyga S, Volpe S, Werthmann RC, Götz K, Sungkaworn T, Lohse MJ, Calebiro D. Persistent cAMP signaling by internalized LH receptors in ovarian follicles. Endocrinology 2016; 157:1613-1621

4. Zhang M, Su YQ, Sugiura K, Xia G, Eppig JJ. Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. Science 2010; 330:366-369

5. Hunzicker-Dunn M, Mayo K. Gonadotrophin signaling in the ovary. In: TM Plant, AJ Zeleznik, eds. Knobil and Neill's Physiology of Reproduction. 4th ed. San Diego: Academic Press; 2015:895-945
6. Jaffe LA, Egbert JR. Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle. Annu Rev Physiol 2017; 79:237-260
7. Richard S, Baltz JM. Preovulatory suppression of mouse oocyte cell volume-regulatory mechanisms is via signalling that is distinct from meiotic arrest. Sci Rep 2017; 7:702
8. Stocco C, Telleria C, Gibori G. The molecular control of corpus luteum formation, function, and regression. Endocr Rev 2007; 28:117-149
9. Czieselsky K, Prescott M, Porteous R, Campos P, Clarkson J, Steyn FJ, Campbell RE, Herbison AE. Pulse and surge profiles of luteinizing hormone secretion in the mouse. Endocrinology 2016; 157:4794-4802
10. McKenna SD, Pietropaolo M, Tos EG, Clark A, Fischer D, Kagan D, Bao B, Chedrese PJ, Palmer S. Pharmacological inhibition of phosphodiesterase 4 triggers ovulation in follicle-stimulating hormone-primed rats. Endocrinology 2005; 146:208-214
11. Jin SL, Richard FJ, Kuo WP, D'Ercole AJ, Conti M. Impaired growth and fertility of cAMP-specific phosphodiesterase PDE4D-deficient mice. Proc Natl Acad Sci U S A 1999; 96:11998-12003
12. Park JY, Richard F, Chun SY, Park JH, Law E, Horner K, Jin SL, Conti M. Phosphodiesterase regulation is critical for the differentiation and pattern of gene expression in granulosa cells of the ovarian follicle. Mol Endocrinol 2003; 17:1117-1130
13. Sasseville M, Albuz FK, Côté N, Guillemette C, Gilchrist RB, Richard FJ. Characterization of novel phosphodiesterases in the bovine ovarian follicle. Biol Reprod 2009; 81:415-425
14. Petersen TS, Stahlhut M, Andersen CY. Phosphodiesterases in the rat ovary: effect of cAMP in primordial follicles. Reproduction 2015; 150:11-20
15. Petersen TS, Kristensen SG, Jeppesen JV, Grøndahl ML, Wissing ML, Macklon KT, Andersen CY. Distribution and function of 3',5'-Cyclic-AMP phosphodiesterases in the human ovary. Mol Cell Endocrinol 2015; 403:10-20
16. Egbert JR, Uliasz TF, Shuhaibar LC, Geerts A, Wunder F, Kleiman RJ, Humphrey JM, Lampe PD, Artemyev NO, Rybalkin SD, Beavo JA, Movsesian MA, Jaffe LA. Luteinizing hormone causes phosphorylation and activation of the cGMP phosphodiesterase PDE5 in rat ovarian follicles, contributing, together with PDE1 activity, to the resumption of meiosis. Biol Reprod 2016; 94:110
17. Bergeron A, Guillemette C, Sirard MA, Richard FJ. Active 3'-5' cyclic nucleotide phosphodiesterases are present in detergent-resistant membranes of mural granulosa cells. Reprod Fertil Dev 2017; 29:778-790
18. Beltejar MG, Lau HT, Golkowski MG, Ong SE, Beavo JA. Analyses of PDE-regulated phosphoproteomes reveal unique and specific cAMP-signaling modules in T cells. Proc Natl Acad Sci U S A 2017; 114:E6240-E6249
19. Schwabe U, Miyake M, Ohga Y, Daly JW. 4-(3-Cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone (ZK 62711): a potent inhibitor of adenosine cyclic 3',5'-monophosphate phosphodiesterases in homogenates and tissue slices from rat brain. Mol Pharmacol 1976; 12:900-910.

20. Vang AG, Ben-Sasson SZ, Dong H, Kream B, DeNinno MP, Claffey MM, Housley W, Clark RB, Epstein PM, Brocke S. PDE8 regulates rapid Teff cell adhesion and proliferation independent of ICER. PLoS One 2010; 5:e12011
21. Soderling SH, Bayuga SJ, Beavo JA. Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase. Proc Natl Acad Sci U S A 1998; 95:8991-8996
22. Sasaki T, Kotera J, Yuasa K, Omori K. Identification of human PDE7B, a cAMP-specific phosphodiesterase. Biochem Biophys Res Commun 2000; 271:575-583
23. Gamanuma M, Yuasa K, Sasaki T, Sakurai N, Kotera J, Omori K. Comparison of enzymatic characterization and gene organization of cyclic nucleotide phosphodiesterase 8 family in humans. Cell Signal 2003; 15:565-574
24. Bian H, Zhang J, Wu P, Varty LA, Jia Y, Mayhood T, Hey JA, Wang P. Differential type 4 cAMP-specific phosphodiesterase (PDE4) expression and functional sensitivity to PDE4 inhibitors among rats, monkeys and humans. Biochem Pharmacol 2004; 68:2229-2236
25. Schmidt CJ, Chapin DS, Cianfrogna J, Corman ML, Hajos M, Harms JF, Hoffman WE, Lebel LA, McCarthy SA, Nelson FR, Proulx-LaFrance C, Majchrzak MJ, Ramirez AD, Schmidt K, Seymour PA, Siuciak JA, Tingley FD, Williams RD, Verhoest PR, Menniti FS. Preclinical characterization of selective phosphodiesterase 10A inhibitors: a new therapeutic approach to the treatment of schizophrenia. J Pharmacol Exp Ther 2008; 325:681-690
26. Shimizu-Albergine M, Tsai LC, Patrucco E, Beavo JA. cAMP-specific phosphodiesterases 8A and 8B, essential regulators of Leydig cell steroidogenesis. Mol Pharmacol 2012; 81:556-566
27. Tsai LC, Beavo JA. Regulation of adrenal steroidogenesis by the high-affinity phosphodiesterase 8 family. Horm Metab Res 2012; 44:790-794
28. Norris RP, Freudzon M, Mehlmann LM, Cowan AE, Simon AM, Paul DL, Lampe PD, Jaffe LA. Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: one of two paths to meiotic resumption. Development 2008; 135:3229-3238
29. Norris RP, Freudzon M, Nikolaev VO, Jaffe LA. Epidermal growth factor receptor kinase activity is required for gap junction closure and for part of the decrease in ovarian follicle cGMP in response to LH. Reproduction 2010; 140:655-662
30. Shuhaibar LC, Egbert JR, Norris RP, Lampe PD, Nikolaev VO, Thunemann M, Wen L, Feil R, Jaffe LA. Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles. Proc Natl Acad Sci U S A 2015; 112:5527-5532
31. Shuhaibar LC, Egbert JR, Edmund AB, Uliasz TF, Dickey DM, Yee SP, Potter LR, Jaffe LA. Dephosphorylation of juxtamembrane serines and threonines of the NPR2 guanylyl cyclase is required for rapid resumption of oocyte meiosis in response to luteinizing hormone. Dev Biol 2016; 409:194-201
32. Teilmann SC, Clement CA, Thorup J, Byskov AG, Christensen ST. Expression and localization of the progesterone receptor in mouse and human reproductive organs. J Endocrinol 2006; 191:525-535
33. Rodriguez KF, Couse JF, Jayes FL, Hamilton KJ, Burns KA, Taniguchi F, Korach KS. Insufficient luteinizing hormone-induced intracellular signaling disrupts ovulation in preovulatory follicles lacking estrogen receptor-β. Endocrinology 2010; 151:2826-2834
34. Jayes FL, Burns KA, Rodriguez KF, Kissling GE, Korach KS. The naturally occurring luteinizing hormone surge is diminished in mice lacking estrogen receptor Beta in the ovary. Biol Reprod 2014; 90:24

35. Breen SM, Andric N, Ping T, Xie F, Offermans S, Gossen JA, Ascoli M. Ovulation involves the luteinizing hormone-dependent activation of G(q/11) in granulosa cells. Mol Endocrinol 2013; 27:1483-1491
36. Tsafriri A, Popliker M, Nahum R, Beyth Y. Effects of ketoconazole on ovulatory changes in the rat: implications on the role of a meiosis-activating sterol. Mol Hum Reprod 1998; 4:483-489
37. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM, O'Malley BW. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev 1995; 9:2266-2278
38. Robker RL, Russell DL, Espey LL, Lydon JP, O'Malley BW, Richards JS. Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. Proc Natl Acad Sci U S A 2000; 97:4689-4694
39. Robker RL, Akison LK, Russell DL. Control of oocyte release by progesterone receptor-regulated gene expression. Nucl Recept Signal 2009; 7:e012
40. Kim J, Bagchi IC, Bagchi MK. Control of ovulation in mice by progesterone receptor-regulated gene networks. Mol Hum Reprod 2009; 15:821-828
41. Tsafriri A, Lieberman ME, Barnea A, Bauminger S, Lindner HR. Induction by luteinizing hormone of ovum maturation and of steroidogenesis in isolated Graafian follicles of the rat: role of RNA and protein synthesis. Endocrinology 1973; 93:1378-1386
42. Park OK, Mayo KE. Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. Mol Endocrinol 1991; 5:967-978
43. Carbajal L, Biswas A, Niswander LM, Prizant H, Hammes SR. GPCR/EGFR cross talk is conserved in gonadal and adrenal steroidogenesis but is uniquely regulated by matrix metalloproteinases 2 and 9 in the ovary. Mol Endocrinol 2011; 25:1055-1065
44. Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, Dekel N. Reactive oxygen species are indispensable in ovulation. Proc Natl Acad Sci U S A 2011; 108:1462-1467
45. Zoccarato A, Surdo NC, Aronsen JM, Fields LA, Mancuso L, Dodoni G, Stangherlin A, Livie C, Jiang H, Sin YY, Gesellchen F, Terrin A, Baillie GS, Nicklin SA, Graham D, Szabo-Fresnais N, Krall J, Vandeput F, Movsesian M, Furlan L, Corsetti V, Hamilton G, Lefkimmiatis K, Sjaastad I, Zaccolo M. Cardiac hypertrophy is inhibited by a local pool of cAMP regulated by phosphodiesterase 2. Circ Res 2015; 117:707-719
46. Shimizu-Albergine M, Van Yserloo B, Golkowski MG, Ong SE, Beavo JA, Bornfeldt KE. SCAP/SREBP pathway is required for the full steroidogenic response to cyclic AMP. Proc Natl Acad Sci U S A 2016; 113:E5685-5693
47. Tsai LC, Shimizu-Albergine M, Beavo JA. The high-affinity cAMP-specific phosphodiesterase 8B controls steroidogenesis in the mouse adrenal gland. Mol Pharmacol 2011; 79:639-648
48. Tsai LC, Beavo JA. The roles of cyclic nucleotide phosphodiesterases (PDEs) in steroidogenesis. Curr Opin Pharmacol 2011; 11:670-675
49. Challiss RA, Adams D, Mistry R, Nicholson CD. Modulation of spasmogen-stimulated Ins(1,4,5)P3 generation and functional responses by selective inhibitors of types 3 and 4 phosphodiesterase in airways smooth muscle. Br J Pharmacol 1998; 124:47-54
50. Palmer D, Tsoi K, Maurice DH. Synergistic inhibition of vascular smooth muscle cell migration by phosphodiesterase 3 and phosphodiesterase 4 inhibitors. Circ Res 1998; 82:852-861

51. Kraynik SM, Miyaoka RS, Beavo JA. PDE3 and PDE4 isozyme-selective inhibitors are both required for synergistic activation of brown adipose tissue. Mol Pharmacol 2013; 83:1155-1165
52. Alaamery MA, Wyman AR, Ivey FD, Allain C, Demirbas D, Wang L, Ceyhan O, Hoffman CS. New classes of PDE7 inhibitors identified by a fission yeast-based HTS. J Biomol Screen 2010; 15:359-367
53. Dong H, Zitt C, Auriga C, Hatzelmann A, Epstein PM. Inhibition of PDE3, PDE4 and PDE7 potentiates glucocorticoid-induced apoptosis and overcomes glucocorticoid resistance in CEM T leukemic cells. Biochem Pharmacol 2010; 79:321-329
54. Carr DW, Cutler RE, Cottom JE, Salvador LM, Fraser ID, Scott JD, Hunzicker-Dunn M. Identification of cAMP-dependent protein kinase holoenzymes in preantral- and preovulatory-follicle-enriched ovaries, and their association with A-kinase-anchoring proteins. Biochem J 1999; 344 Pt 2:613-623

Figure 1. PDE4, PDE7, and PDE8 act together to suppress premature cAMP elevation in preovulatory follicles. **A**) Time course of cAMP elevation after exposure of follicles to various concentrations of LH, or to a mixture of inhibitors of 3 cAMP phosphodiesterases: PDE4i (rolipram, 5 μM), PDE7i (1 μM), and PDE8i (PF04957325, 1 μM). The LH data were obtained from the indicated number of independent experiments, each including all time points. The PDE inhibitor data includes 2-4 measurements for each time point. T-tests were performed to test the statistical significance of the difference between the measurements following no LH and 1 nM LH treatments; the black asterisk indicates a significant difference ($p<0.05$ after correction for multiple comparisons). T-tests were also used to test the difference between measurements following no LH and PDE inhibitor treatments; green asterisks indicate significant differences. **B**) cAMP content of follicles after a 4-hour exposure to each of the inhibitors listed in A, or to a mixture of the 3 inhibitors. Here and in subsequent figures, different letters indicate significant differences ($p<0.05$).

Figure 2. PDE4, PDE7, and PDE8 act together to suppress premature nuclear envelope breakdown in follicle-enclosed oocytes. **A**) A follicle prior to exposure to LH or a PDE inhibitor; an intact nucleus is visible in the oocyte. **B**) Time course of NEBD in response to various concentrations of LH. **C**) Percent NEBD at 6 hours after treatment of follicles with a saturating concentration of LH (300 nM), individual phosphodiesterase inhibitors, pairs of inhibitors, or all three inhibitors together (5 μM rolipram, 1 μM PDE7 inhibitor, 1 μM PDE8 inhibitor). **D**) Similar percent NEBD in response to 5 and 20 μM rolipram. **E**) Time course of NEBD in response to LH (300 nM), individual PDE inhibitors (concentrations as in C), or a mixture of the 3 inhibitors.

Figure 3. PDE4, PDE7, and PDE8 act together to suppress premature ovulation. **A**) A follicle at 24 hours after exposure to LH (300 nM). Ovulation has occurred, as indicated by extrusion of the oocyte and some granulosa cells. **B**) Percent ovulation at 24 hours after exposure to various concentrations of LH. **C**) Percent ovulation at 24 hours after treatment of follicles with LH (300 nM), individual phosphodiesterase inhibitors, pairs of inhibitors, or all three inhibitors together (5 μM rolipram, 1 μM PDE7 inhibitor, 1 μM PDE8 inhibitor). **D**) Similar percent ovulation in response to 5 and 20 μM rolipram. **E**) A follicle at 24 hours after treatment with a mixture of inhibitors of PDEs 4, 7, and 8 (concentrations as in C), showing ovulation as seen with LH.

Figure 4. PDE4, PDE7, and PDE8 all contribute to suppressing spontaneous production of progesterone and progesterone receptors. **A**,**B**). Time course of synthesis of progesterone (**A**) and its receptors PGR-A and PGR-B (**B**) in response to LH (300 nM). **C**-**F**). Stimulation of synthesis of progesterone (**C**) and progesterone receptors (**D**-**F**) by incubation of follicles for 6 hours in the presence of LH (300 nM) or inhibitors of PDE4 (rolipram, 5 μM), PDE7 (1 μM) and/or PDE8 (1 μM). In panels B and D, the doublet near the M_r 97 marker corresponds to the PGR-A isoform, and the doublet just above the M_r 116 marker corresponds to the PGR-B isoform; the doublets are thought to result from phosphorylation (32). The lower bands, in the range of M_r ~50-70 are unidentified, but have been seen in a previous study as well (32).

Figure 5. LH signaling does not cause a rapid decrease in cAMP phosphodiesterase activity. cAMP phosphodiesterase activity was measured in lysates of follicles with or without a 30 minute exposure to LH (300 nM). Activity was assayed with 2 different cAMP substrate concentrations, 0.1 μM or 1 μM. Bars show the mean ± s.e.m. for 2 independent experiments.

Table 1. Selectivity data for the PDE7 and PDE8 inhibitors. Assays were performed using recombinant human phosphodiesterase proteins, except for PDE6, which was extracted from bovine retina. Table 1A lists mean values for the dissociation constant of the enzyme-inhibitor complex (K_i) for the PDE7 inhibitor (PDE7i) and for the PDE8 inhibitor (PDE8i), determined for the indicated phosphodiesterases and substrates. Tables 1B and 1C list mean ± s.e.m. values for pKi (the negative of the base 10 logarithm of the Ki), testing PDE7i (Table 1B) and PDE8i (Table 1C) with the indicated phosphodiesterases. N indicates the number of assays. Mean IC_{50} values for PDE8i inhibition of PDE8A and PDE8B were previously reported to be 0.7 and <0.3 nM respectively (20; substrate concentration not specified) and 3.1 and 0.4 nM respectively (27; 12-14 nM cAMP substrate).

Table 1A

		PDE7i	PDE8i
PDE	substrate	K_i (nM)	K_i (nM)
PDE1B1	cAMP	7200	>9900
PDE2A1	cGMP	>9900	>9900
PDE3A1	cAMP	>9800	>9800
PDE4D3	cAMP	3800	3000
PDE5A1	cGMP	>9600	>9600
PDE6	cGMP	>9900	>9900
PDE7B	cAMP	7.8	2500
PDE8B	cAMP	>9500	0.1
PDE9A1	cGMP	>9600	>9600
PDE10A1	cAMP	>9300	>9300
PDE11A4	cGMP	>9200	>9200

Table 1B: PDE7i

PDE	pK_i (mean ± s.e.m.)	K_i (nM)	N
PDE7B	8.11 ± 0.05	7.8	6
PDE1B1	5.14 ± 0.16	7200	4
PDE4D3	5.42 ± 0.24	3800	4

Table 1C: PDE8i

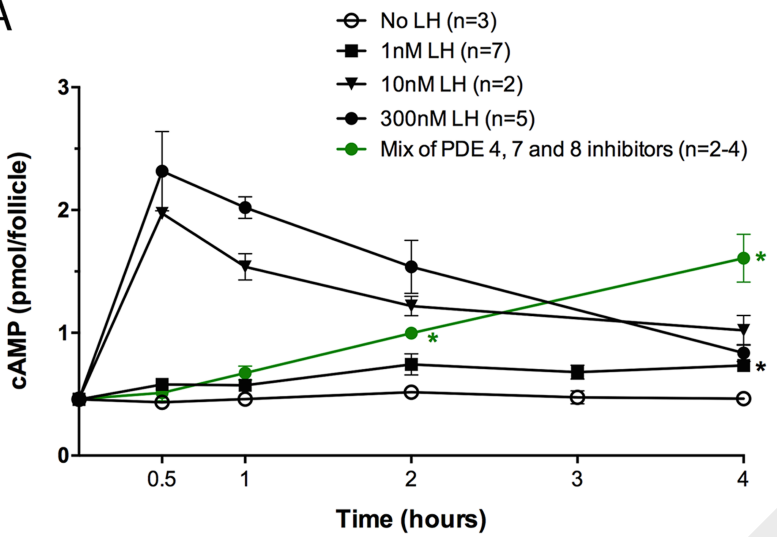
PDE	pK_i (mean ± s.e.m.)	K_i (nM)	N
PDE8B	8.74 ± 0.06	0.1	3
PDE7B	5.60 ± 0.14	2500	4

PDE4D3	5.52 ± 0.05	3000	4

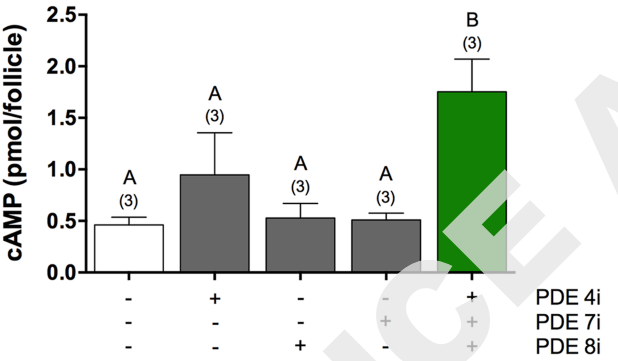
Table 2. Antibody descriptions

Protein Target	Antigen	Name of Antibody	Manufacturer, Catalog Number	Species, Monoclonal/Polyclonal	Dilution Used	RRID
Progesterone receptor, PRG-A and PRG-B isoforms	Amino acids 412-526 of human progesterone receptor	Clone SP2	Abcam, ab 16661	Rabbit, monoclonal	1:500	AB_443421
Rabbit IgG	Rabbit IgG (heavy and light chains)	Goat Anti-Rabbit IgG, IRDye® 800CW Conjugated antibody	Licor, 926-32211	Goat, polyclonal	1:15000	AB_621843

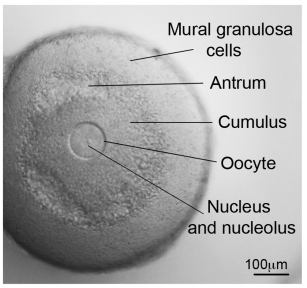
A



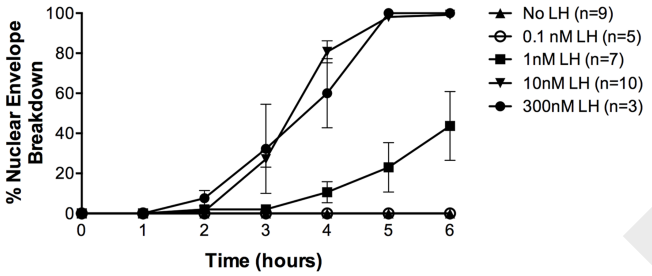
B



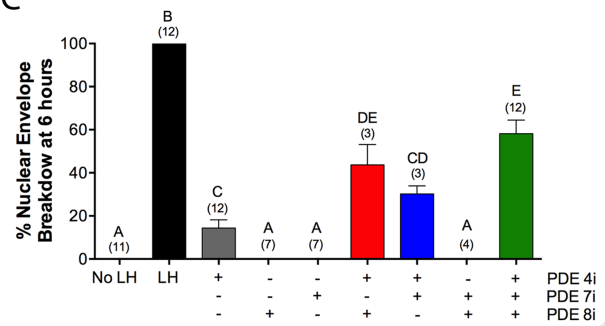
A



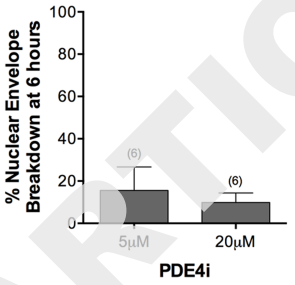
B



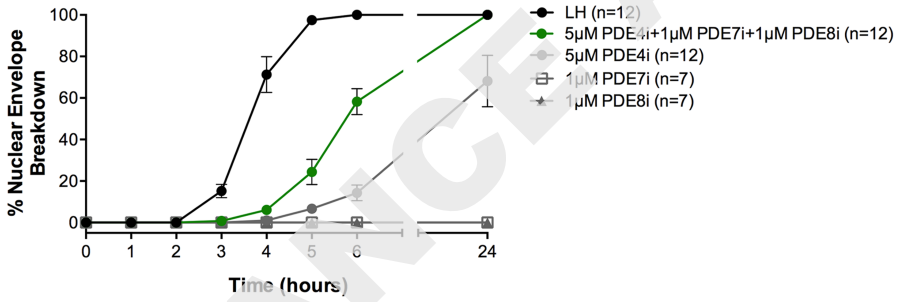
C



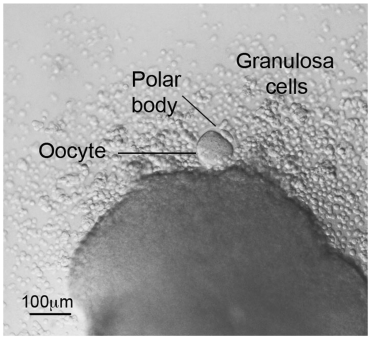
D



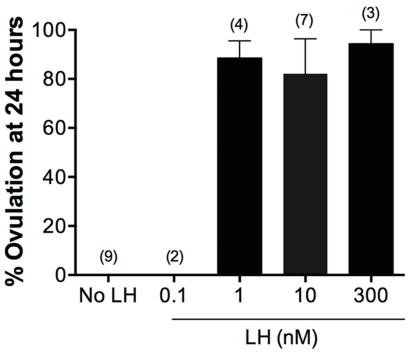
E



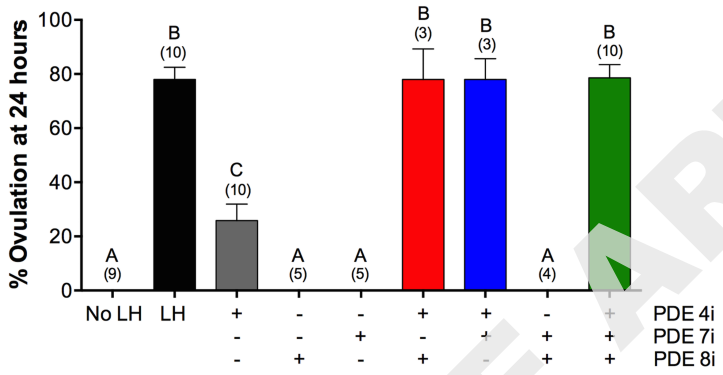
A



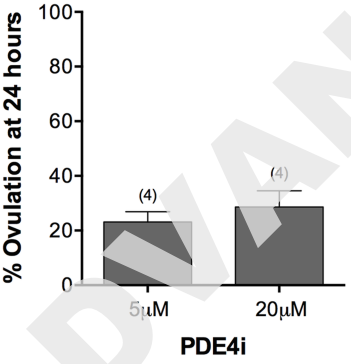
B



C



D



E