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## SUPPLEMENTARY MATERIAL

## SUPPLEMENTARY TABLES S1-S2

Table S1. Primers and fluorescent probes used for qRT-PCR analysis of relative expression levels of cyclic nucleotide phosphodiesterases in rat mural granulosa cells.

| Gene | Forward primer, 5'-3' | [a]Probe, 5'-3' | Reverse primer, 5'-3' |
|---|---|---|---|
| *Pde1a* | ACAGAGCCAAAACGATGTCC | TGATACTCCACGCAGCCGACATCAG | CACCTGTAGTGCAGCTTCCA |
| *Pde1b* | AATGCGCCATCCTGTACAATGA | CGATCGGTGCTGGAGAATCACCA | CATCATTCGGAAAACAGAGCTGAT |
| *Pde1c* | GCCTAGCTGCTGAGGAAAAG | AAGCCAAAAGCCAAGCTGAACAAGG | TTTCAGCTTTGCTGGTTGTG |
| *Pde2a* | GAGATGATGGATCGGGAGAA | CCTACATCCCTGAACTTCAGATCAGCTT | CATGGCGATGTGCTCCATA |
| *Pde3a* | CTGCAGCATTCCTCAGAACA | TCCAGGCTATCAAGGAGGAGGAGGA | CTCTTGGCTTCCCCTTCTCT |
| *Pde3b* | CCCCCTGTGCAACTCCTAT | CTGGCTTGCTGCCTGGTCAGTG | CGTCACCCTCTTCTGCTTCT |
| *Pde4a* | GAACTGGAGAACTTGAGCAAATG | TGAACATCTTTTGTGTGTCGGAGTACG | GAGTGAGCGGCCTCCAG |
| *Pde4b* | GGGAGAAGGCCCCAACTAT | TCAGCAGCACAAAGACACTTTGTGTGA | GAATCCCTGTTCTCTGGATC |
| *Pde4c* | CCACACCAGCCTTCCTACAG | ATCCCGCGCTTTGGGGTCC | CTGCTCCTCCTGGTCTGTCT |
| *Pde4d* | GAAGAAAAGGCCGATGTCAC | AGTTGATGCACAGCTCCAGCCTGAC | ATCTTGGAATGCAGGAATTG |
| *Pde5a* | GTCTGCCCAAACCCTTAAAA | CCGACTTCAGCTTCAGTGACTTCGA | CGCTGTTTCCAGATCAGACA |
| *Pde6a* | CCATGTTGGATGGGATCACT | ACAACCGTAAGGAGTGGAAGGCGCT | CATCCTGGCCTCGTACTCAT |
| *Pde6b* | CTCGATTTCACGAAGAGATCC | CCCATGTTTGACCGACTGCAAAATA | TCAGCTAGAGCTTTCCACTCC |
| *Pde6c* | CTGACCGGCCTTCAGAATAA | AGAGTGGAATGGAAATCACTGGCGG | TCACCTTCGCCTCATACTCC |
| *Pde7a* | GATGGAGGCTCAGATAGGTG | TTGATACTAGCCACGGACATCAGTCG | CAATGACAGGTACTCATTCTGG |
| *Pde7b* | CCACGGACATCAACAGACAG | TTCCTGACCCGCTTAAAAGCTCACC | CCAGTCTCAAATCCTTATTGTGG |
| *Pde8a* | GGAAAGGACTGGATGAGAAGG | CTCCGGCCCCCTCCAGAATAGTG | AGGGGGCCTAGTGTGTACCT |
| *Pde8b* | ACACCTGCCAGCCCTGAT | CAACACCTGGCCGACAACTATAAACAC | TTTGCACTTGAGGTCATCCA |
| *Pde9a* | CATGGACCGAGACAAAGTGA | AAGCGACAGCCCAAATTGGGTTCAT | TCAAACATTGGGATTAGGACAA |
| *Pde10a* | GAGGGGAAGAGACAGCAATG | TTCAGGCCCAGCAACTAGCAAAAGC | CAGTCATCGACCTTCCTGGT |
| *Pde11a* | GACCGGAACCGGAAAGAT | CTGCCTCGGCTGCAACTGGAGT | CATGCAGATGCTGTCAATCC |
| *L32* | TTACTGTGCTGAGATTGCTCAC | TGTGTCCTCTAAGAACCGAAAAGCCA | CCAGCTGTGCTGCTCTTTC |

[a]Probes were labeled with FAM (5') and TAMRA (3').

Table S2. Primers and fluorescent probes used for qRT-PCR analysis of relative expression levels of cyclic nucleotide phosphodiesterases in mouse mural granulosa cells.

| Gene | Forward primer, 5'-3' | [a]Probe, 5'-3' | Reverse primer, 5'-3' |
|---|---|---|---|
| *Pde1a* | GTCCCTGATACTCCATGCAG | TGACATCAGCCACCCAGCCAAAACT | CCATCTGTAGTGCAACTTCC |
| *Pde1a_N1* | GCCCATAATGATGTTCCTAGGA | ACAGGTGCAGTCAGTTTCAGCGGCT | TGCAATGGACTGAGGAATGC |
| *Pde1a_N2* | GTTTTCTCCCAGCACCCAAA | TCCTTCTGGGATCCTTGCAGCACAA | GGGAGGGAATGTGGAAGCTA |
| *Pde1a_N3* | ACAATGGCTCCTGCTCCAC | CCCTGCTCTGTAGATGTGGGGGATTGC | CTATACTACAAGCCTTGACATCTTC |
| *Pde1b* | AATGTGCCATCCTGTACAATGA | CGATCGGTGCTGGAGAATCACCA | CATCATTCGAAAGACAGAGCTGAT |
| *Pde1c* | TTCTGGGTCAGATGGAAGTG | CCCATCAACAATTCTGTCATTCCTGTT | ACCTCAGTCCAAGTGGCTTT |
| *Pde2a* | ATCCTGAACATCCCAGATGC | CCCATCCGCTTTTCTATCGCGG | AGGGAAGCAGAGAATGTTGC |
| *Pde3a* | AGAGCAGATCCAGGCTATCAA | AGGAAGAGGAAGAGAAGGGGAAGCC | CCAGGGTCTCCTCTGCTCTT |
| *Pde3b* | TGGATCGTTCTTCTCCTCAA | CTTTTATCACCCACATTGTGGGCCC | CAGCATCATAGGAGTTGCACA |
| *Pde4a* | CAAATGGGGCCTGAACAT | TTTGTGTGTCGGAGTACGCTGGAGG | ACATGATACAGCTGAGTGAACG |
| *Pde4b* | GGGAGAAGGCCACAGCTAT | TCAGCAGCACAAAGACGCTTTGTGTGA | GAATCCCTGTTCTCTGGATC |
| *Pde4c* | CCACACCAGCCTTCCCACAG | ATCCCACGCTTTGGGGTCC | CTGCTCCTCCTGATCTGTCT |
| *Pde4d* | CTCGGAACTCGCTCTGATGT | CAACGACTCCTCGGTCCTAGAGAACCATCA | ACTTAAAGCCCACCGCCAAG |
| *Pde5a* | CCGACTTCAGCTTCAGTGACTT | TGTCTGATCTGGAAACAGCGCTGTG | GGTCAGTGAACATCCGAATTG |
| *Pde6a* | CATGCTGGATGGGATCACTA | AAGGAATGGAAGGCGCTGGCTGAT | GCCTTCATCTTGGCTTCGTA |
| *Pde6b* | TCGATTTCACGAAGAGATCC | CCCATGTTTGACCGACTGCAAAATA | TCGTCAGCTAGTGCTTTCCA |
| *Pde6c* | TCGGTTTCATGGAGAGATCA | CCCATGCTGAACGGCCTTCAGAATA | GGATTTCCATTCCACCCTGT |
| *Pde7a* | TGGAGGCTCAGATAGGTGCT | CACGGATATCAGTCGCCAGAACGAG | CCAAGTGAGATCTAAACAATGACA |
| *Pde7b* | CTGGGCTCCCTCATCTTG | CAGACAGAATGAGTTTCTGACCCGC | TCTTTATTGTGGAGGTGAGCTTT |
| *Pde8a* | TCGTTGACCTGCCTAACCTT | TGCAGCACCTAGATGACAACTTCAGG | TTCGAAGCTTCTTCTCATCCA |
| *Pde8b* | CTGGATGACCTCAAGTGCAA | CTTCCATCTGACAGCTGAAGCCACG | TCACAGTGTCCCCTTCTCG |
| *Pde9a* | CATGGACCGAGACAAAGTGA | AAAGCAACAGCCCAAATTGGGTTCA | TGGGATCAGGACAAACTTGA |
| *Pde10a* | GAGCAGGTGCTGGAGATCAT | CGCAAAGCCATCATCGCCAC | TCCTCCAACTGCTTCCTGTT |
| *Pde11a* | TTTTCCCTTGATGTTGATGC | TCACAGCCGCTCTACGGATGTTCAT | TTCTGTACCATCCCCAGCTC |
| *L32* | ATTGTAGAAAGAGCAGCACAGC | CCAGGCTACGCAGCGAAGAAAATGA | AAACATGCACACAAGCCATC |

[a]Probes were labeled with FAM (5') and TAMRA (3').

## SUPPLEMENTARY FIGURES S1-S7

Figure S1.

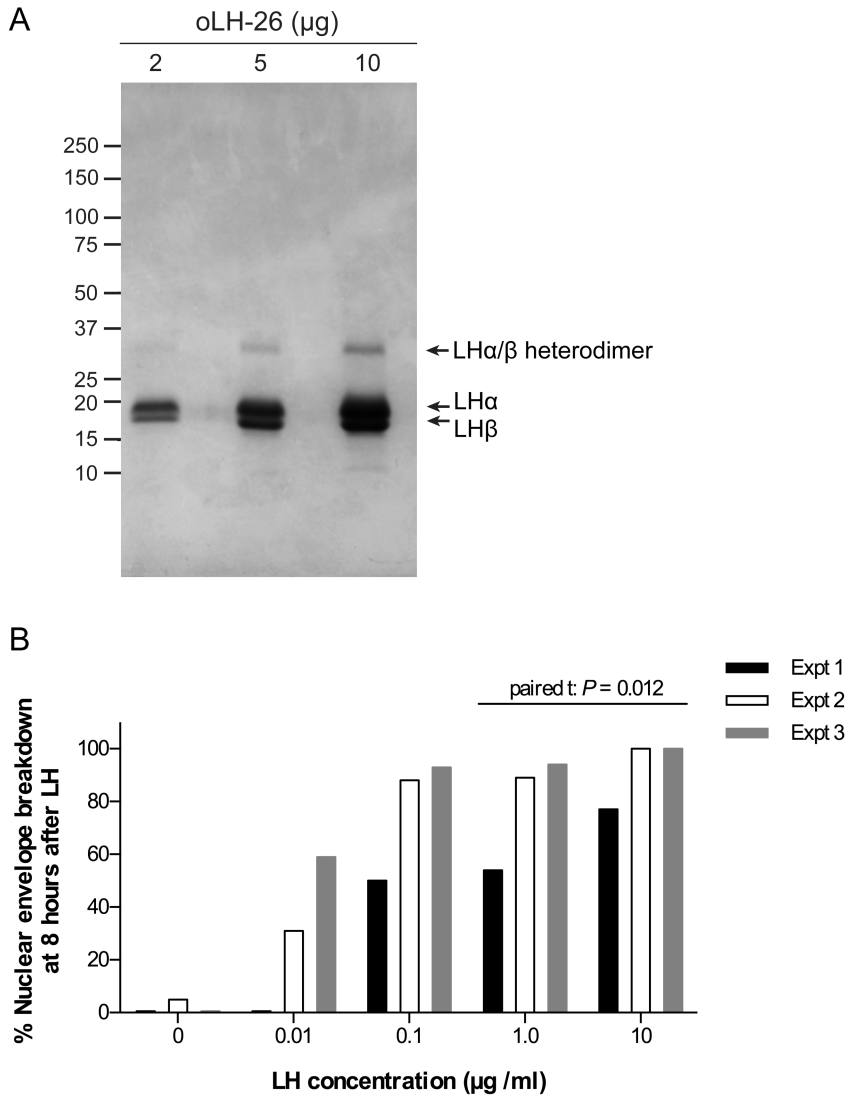


FIG. S1. Purity of oLH-26 as indicated by SDS-PAGE, and the concentration dependence of its ability to stimulate meiotic resumption in isolated rat ovarian follicles. **A**) A Coomassie-stained gel (4-20% gradient) of various amounts of oLH-26. Bands were identified as the LH α and β subunits, and as the LH heterodimer, by comparison with previously published gels [71]. The faint band at ~10 kDa is unidentified but may result from proteolysis of the β-subunit [71]. **B**) Isolated rat follicles were cultured for 8 hours in the presence of various concentrations of oLH-26. At the end of the culture period, oocytes were isolated and scored for NEBD. Bars represent results obtained in 3 separate experiments. For each experiment, 13-20 follicles were scored at each concentration. Some variability in sensitivity to LH was seen among the 3 experiments, but in all 3 the response was maximal at 10 µg/ml LH. A paired t-test showed that the % NEBD was significantly greater at 10 µg/ml vs. 1 µg/ml ($P = 0.012$, data was arcsine-square root transformed).

Figure S2.

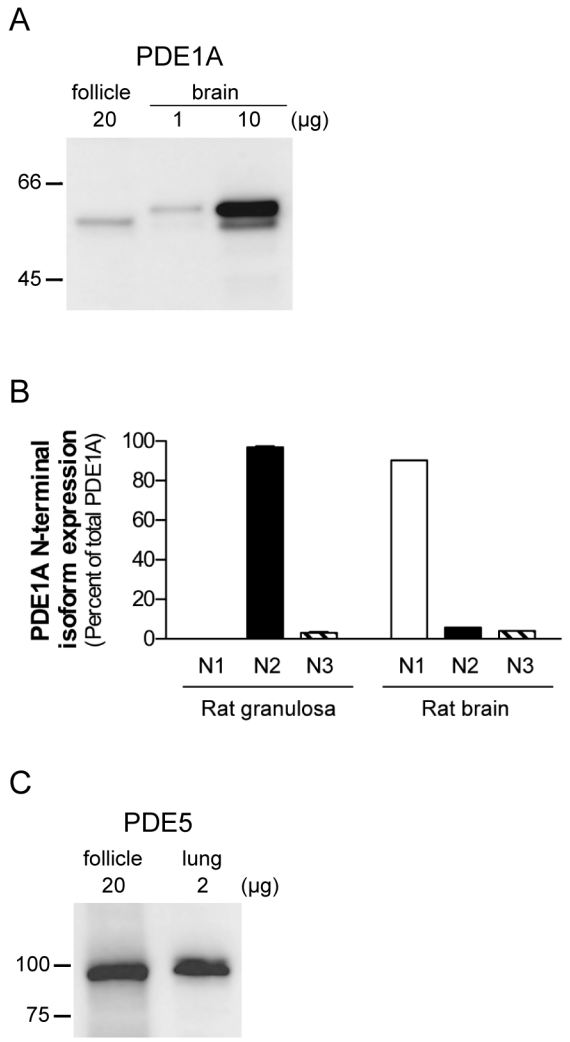


FIG. S2. Western blots comparing PDE1A and PDE5 in rat follicles and control tissues. **A**) The level of PDE1A in rat follicles is ~10% of that in rat brain (hippocampus). The molecular weight of PDE1A in follicles is slightly smaller than that of the main form of PDE1A in brain, and similar to that of a minor form. **B**) Correspondingly, qRT-PCR showed that the main splice variant of PDE1A in granulosa cells is N2, vs N1 in brain; N2 is 18 amino acids shorter than N1 [72]. **C**) The level of PDE5 in rat follicles is ~10% of that in lung.

Figure S3.

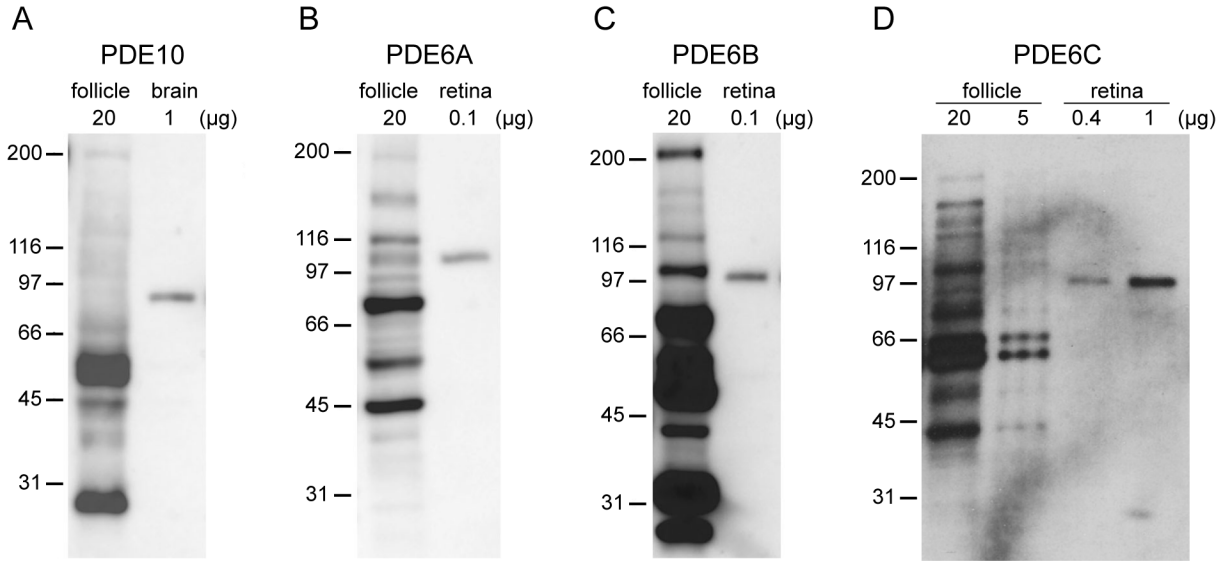


FIG. S3. Western blots showing little or no PDE10, PDE6A, PDE6B, and PDE6C proteins in rat follicles. **A**) PDE10 was detected in rat brain (caudate nucleus, 1 µg protein), but no distinct band that comigrated with brain PDE10 was detected in rat follicles (20 µg protein). Based on the limit of detectability, any PDE10 protein in the follicle was present at ≤ 2% of the level in brain. Rat PDE10 has several splice variants, but the predicted molecular weights of these are larger than that of the major form present in brain [73]. PDE10 was detected using mouse monoclonal antibody 24F3.F11 that was produced using full-length rat PDE10 recombinant protein as the antigen [74]. This antibody was a gift from Timothy Coskran (Pfizer, Inc., Groton, CT). **B,C,D**) PDE6A, PDE6B, and PDE6C proteins were detected in mouse retina, but bands of similar intensity at similar molecular weights were only seen when the amount of follicle protein was 50-200 times that loaded in the retina lane. These results indicated that the levels of these PDE6 proteins in follicles are ≤ 2% of those in retina. PDE6A and PDE6B were detected using rabbit polyclonal antibodies from Proteintech (Chicago, IL) (122442-2-AP, 21200-1-AP, and 22063-1-AP). PDE6C was detected using a rabbit polyclonal antibody described by [75], PDE6C-157-175. Similar results were obtained with 2 other PDE6A antibodies, made against different antigens (Pierce/Thermo PA-720, and PDE6A-31-50 as described by [75]. In the regions of the antigens used to make these antibodies, the rat and mouse sequences are ≥95% identical, and the rat and mouse sequences each contain the same number of amino acids. Therefore, mouse retina could be used as a positive control for rat follicles. Splice variants have not been described for PDE6A-C [76].

Figure S4.

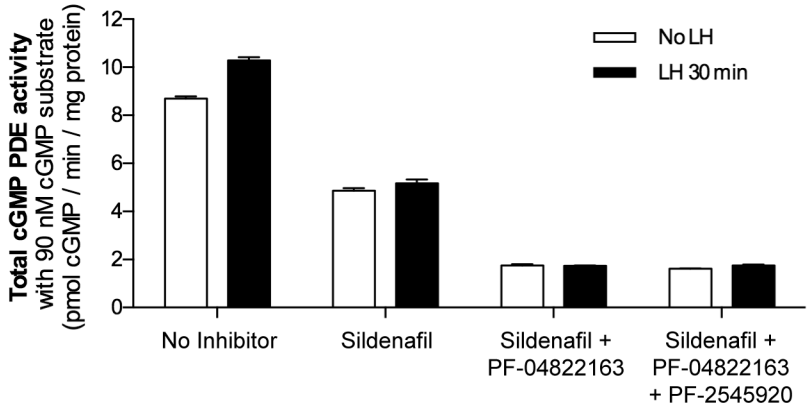


FIG. S4. Addition of a PDE10 inhibitor (PF-2545920, 100 nM) to rat follicle lysates did not reduce the residual cGMP-hydrolytic activity seen in the presence of inhibitors of PDE5 (sildenafil, 100 nM) and PDE1 (PF-04822163, 100 nM). The graph represents results from a single set of follicle samples, assayed in duplicate. Error bars represent the s.e.m. of the two duplicates.

Figure S5.

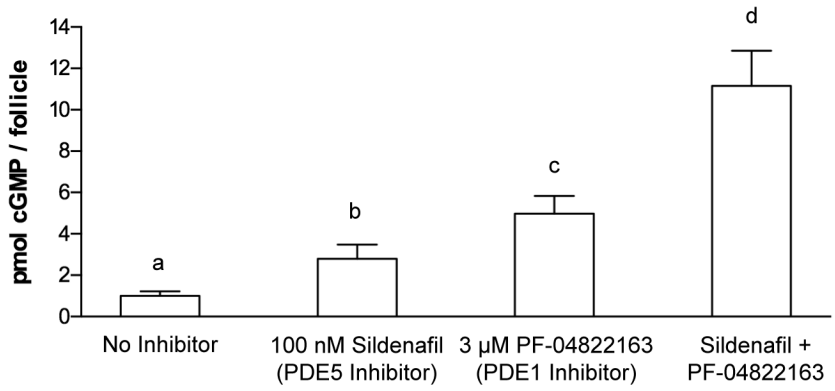


FIG. S5. Incubation of rat follicles with a PDE5 inhibitor and/or a PDE1 inhibitor elevated their cGMP content. cGMP was measured using an ELISA as previously described [18]. The graph shows the mean ± s.e.m. for 7 sets of follicle samples. Data were analyzed by repeated measures one-way ANOVA followed by Tukey correction for multiple comparisons. Different letters indicate significant differences ($P < 0.05$).

Figure S6.

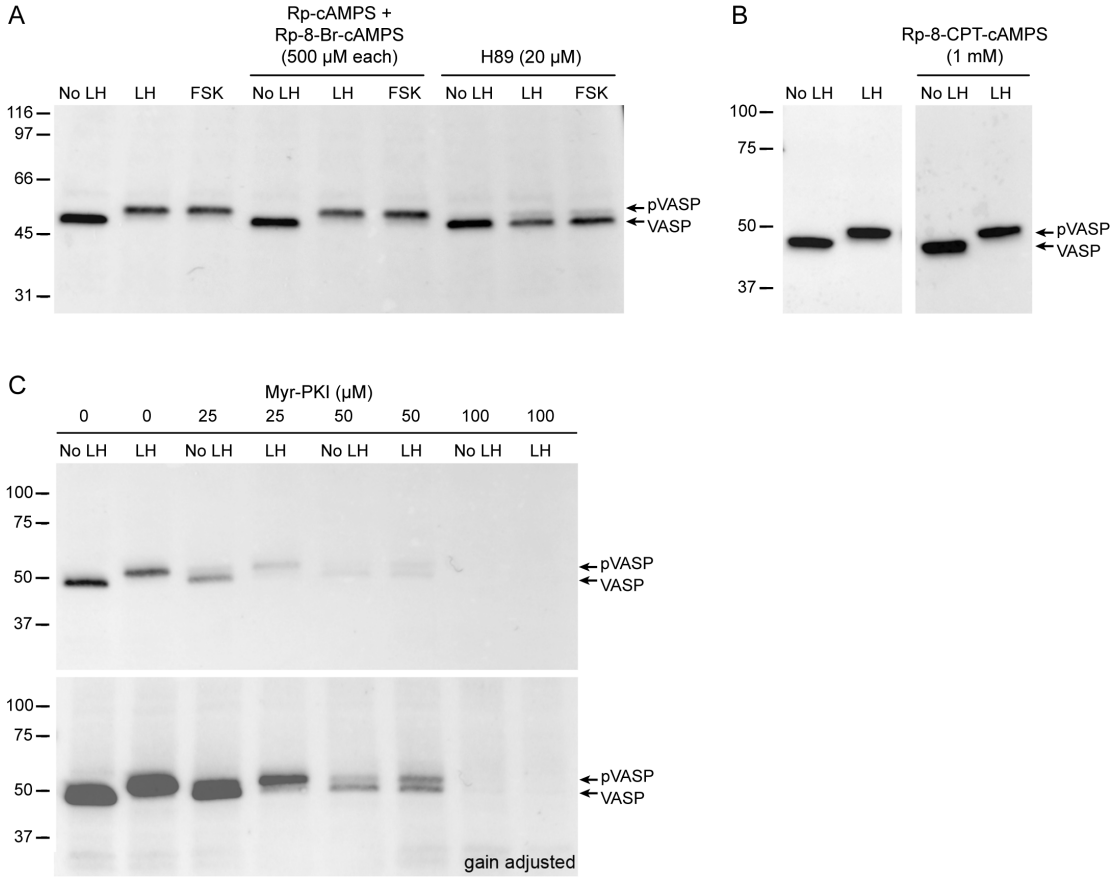


FIG. S6. Lack of effect of inhibitory cAMP analogs or a myristoylated PKI peptide on LH- or forskolin-induced increases in protein kinase A (PKA) activity in rat granulosa cells. PKA activity was detected by phosphorylation of VASP, as indicated by a shift in migration in an SDS-PAGE gel. VASP can be phosphorylated by PKA [77, 78] as well as other kinases [79], but since elevation of cAMP by forskolin stimulates PKA, the gel shift could be used to monitor PKA activity. To improve permeability of the inhibitors, we used isolated mural granulosa cells in culture, since these cells show LH-induced PDE5 phosphorylation like that seen with intact follicles (Fig. 4F). Cells were preincubated with inhibitors for one hour, then treated for 30 min with LH or 10 μM forskolin. **A**) Rp-cAMPS + Rp-8-Br-cAMPS (500 μM each) had no effect, but H89 (20 μM) prevented LH- and forskolin-induced phosphorylation of VASP. **B**) Rp-8-CPT-cAMPS, 1 mM, was also ineffective. **C**) Myristoylated PKI 14-22 amide, 25-100 μM, was toxic at all concentrations tested, based on the appearance of the cells; higher concentrations were more toxic, and VASP protein loss from the cells was seen, indicating that the plasma membrane was leaky. 25 μM Myr-PKI did not inhibit LH-induced phosphorylation. 50 μM Myr-PKI caused some increase in basal phosphorylation of VASP, and had some inhibitory effect on the increase in phosphorylation in response to LH or forskolin (see higher gain image); however, this concentration of Myr-PKI caused loss of most of the VASP protein in the cells. VASP was detected using a rabbit monoclonal antibody from Cell Signaling Technology (3132).

Figure S7.

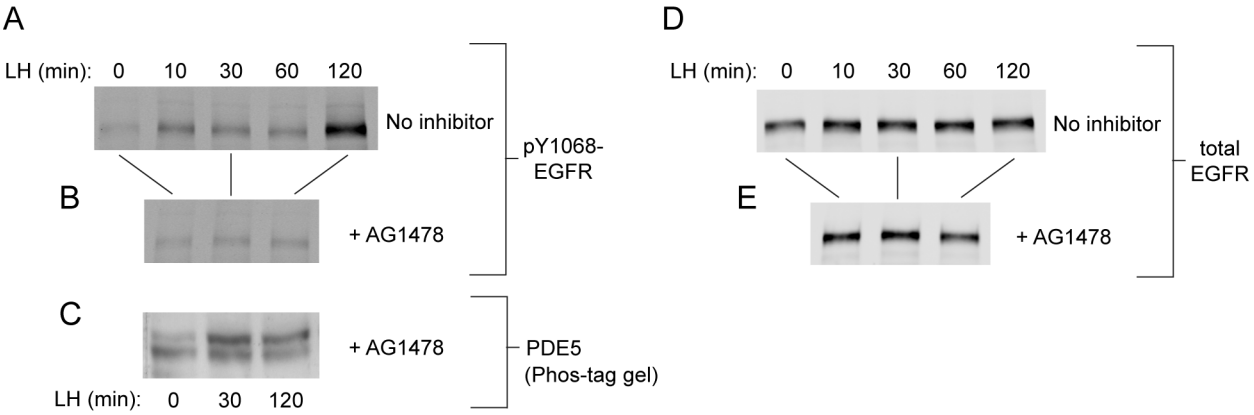


FIG. S7. Inhibition of the LH-induced phosphorylation of EGFR does not inhibit LH-induced phosphorylation of PDE5 in rat follicles. **A**) Time course of EGFR phosphorylation in response to LH. A western blot of follicles treated for various times with LH was probed with a pY1068-EGFR phosphospecific antibody (Cell Signaling Technology, 3777). **B**) AG1478 (1 μM, 1 hour preincubation) effectively inhibits LH-induced phosphorylation of EGFR. AG1478-treated follicles were prepared and processed in parallel with samples used for **A**. **C**) LH-induced PDE5 phosphorylation is not blocked by AG1478, indicating that EGFR signaling does not mediate this process. The same rat follicle lysates used for B were separated on a Phos-tag-containing gel and probed with a total PDE5 antibody; the upper bands represent phosphorylated PDE5. These results are similar to those obtained with samples without AG1478; see Fig. 4D. Two other independent experiments also showed that 1 μM AG1478 did not inhibit LH-induced phosphorylation of PDE5 at 30 or 120 min. **D**,**E**) Blots in **A,B** were stripped and reprobed with a total EGFR antibody (Upstate/EMD Millipore 06-847) to confirm equal loading of lanes.

## SUPPLEMENTARY REFERENCES

71. Bousfield GR, Ward DN. Selective proteolysis of ovine lutropin or its $\beta$ subunit by endoprotease Arg-C: properties of the $Arg^{\beta 43}$ cleaved hormone. J Biol Chem 1988; 263:12602-12607.

72. Michibata H, Yanaka N, Kanoh Y, Okumura K, Omori K. Human $Ca^{2+/}$calmodulin-dependent phosphodiesterase PDE1A: novel splice variants, their specific expression, genomic organization, and chromosomal localization. Biochim Biophys Acta 2001; 1517:278-287.

73. O'Connor V, Genin A, Davis S, Karishma KK, Doyere V, De Zeeuw CI, Sanger G, Hunt SP, Richter-Levin G, Mallet J, Laroche S, Bliss TVP, French PJ. Differential amplification of intron-containing transcripts reveals long term potentiation-associated up-regulation of specific Pde10A phosphodiesterase splice variants. J Biol Chem 2004; 16:15841-15849.

74. Seeger TF, Bartlett B, Coskran TM, Culp JS, James LC, Krull DL, Lanfear J, Ryan AM, Schmidt CJ, Strick CA, Varghese AH, Williams RD, Wylie PG, Menniti FS. Immunohistochemical localization of PDE10A in the rat brain. Brain Res 2003; 985:113-126.

75. Majumder A, Pahlberg J, Muradov H, Boyd KK, Sampath AP, Artemyev NO. Exchange of cone for rod phosphodiesterase 6 catalytic subunits in rod photoreceptors mimics in part features of light adaptation. J Neurosci 2015; 35:9225-9235.

76. Omori K, Kotera J. Overview of PDEs and their regulation. Circ Res 2007; 100:309-327.

77. Eigenthaler M, Nolte C, Halbrügge M, Walter U. Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. Eur J Biochem 1992; 205:471-481.

78. Halbrügge M, Eigenthaler M, Polke C, Walter U. Protein phosphorylation regulated by cyclic nucleotide-dependent protein kinases in cell extracts and in intact human lymphocytes. Cell Signal 1992; 4:189-199.

79. Wentworth JKT, Pula G, Poole AW. Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated on $Ser^{157}$ by protein kinase C-dependent and –independent mechanisms in thrombin-stimulated human platelets. Biochem J 2006; 393:555-564.