SUPPLEMENTARY MATERIAL

Table S1. Primers and fluorescent probes used for qRT-PCR analysis of relative expression levels of PPP family phosphatases.

gene name	forward primer, 5'-3'	probe, 5'-3'	reverse primer, 5'-3'
Ppp1ca	AAGGGGAAGTATGGGCAGTT	AGGCCGTCCCATCACTCCACC	TTTCTTGGCTTTGGCAGAAT
Ppp1cb	ACCAGTATGGTGGGCTGAAT	CTGTCACTCCGCCTCGAACAGCTAA	TTCACCTTTTCTTCGGTGGA
Ppp1cc	CCCAACTACTGTGGCGAGTT	AATGCGGGCGCCATGATGAGT	AGCACATGAGGGTCTCATCC
Ppp2ca	CGTAGAGGCGAGCCACA	CTCGTCGTACCCCAGACTACTTCCTG	TTCATGGCAATACTGTACAAGG
Ppp2cb	CGTCGTGGAGAGCCTCAT	CCGGCGCACCCCAGACTACTTC	AGGTCCTGGGGAGGAATTTA
Ppp3ca	ACGCCAACCTTAACTCCATC	TCGCCTCAGAGACTAACGGCACAGA	TGCTGCTATTACTGCCGTTG
Ppp3cb	AGGAGAGTGAAAGCGTGCTG	AAGGGCCTGACTCCCACAGGGAT	CCAGCCAACACTCCACTAGG
Ppp3cc	TTGAAGAAGCCCGAGGTCTA	TGAGAGAATGCCACCCCGAAAAGAG	GTGTCTTTCCTGCATCATGG
Ppp4c	GGCAGCCATCTTAGAACTGG	TTCATCATCTTCGAGGCTGCACCC	GGATGCCACGTGTCTCTTG
Ppp5c	GACCAGATGGGAAACAAAGC	ACATCCACCTCCAGGGCTCCGAC	CACTGCTGTGAATTGGTGGA
Ppp6c	CGTCAACACGAGAGAACCAA	TCCGAGCAGTTCCAGATTCAGAACG	CGTGGTTCTGGGAGGAATAA
Ppef1	TGTGGAAACTTTTCAATGCTCA	ATGATTCCCAAATTGATGAGCTTGCC	TTGTTGGAGTCCATTGTGCT
Ppef2	CATCTGTGACCTTGCCAGAA	TTCAACAAGGACGGCCACATCGATA	AAGGCCTCCAGGAACTCATT
Rpl32	GAAAGAGCAGCACAGCTGGC	TCAGAGTCACCAATCCCAACGCCA	TCATTCTCTTCGCTGCGTAGC

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

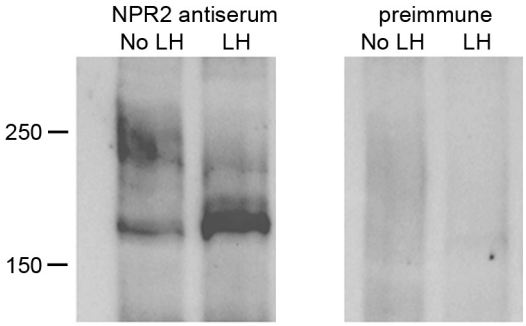

Figure S1. Validation of the specificity of the NPR2 antibody. Blot showing the lack of immunoreactivity when the immunoprecipitation and western blot of follicle membranes were done with preimmune serum instead of the 6328 antibody against a C-terminal peptide from NPR2. The follicles were treated with or without LH for 30 minutes.

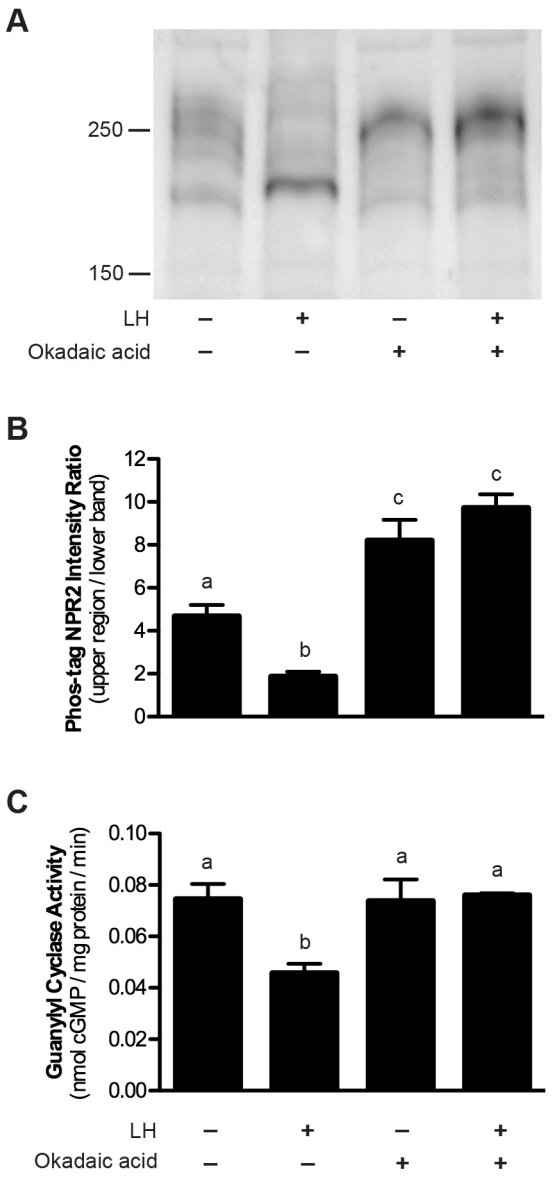

Figure S2. Inhibition of the LH-induced dephosphorylation and inactivation of NPR2 by treatment of follicles with the PPP family phosphatase inhibitor okadaic acid. (A) Follicles were incubated with or without 10 μM okadaic acid for one hour, then with or without LH for 30 minutes. Crude membranes were isolated and used for immunoprecipitation, Phos-tag gel electrophoresis, and immunoblotting for NPR2. In the presence of okadaic acid, basal phosphorylation of NPR2 increased, but LH did not change the ratio of NPR2 in the upper region and lower band, indicating that okadaic acid inhibited the LH-induced dephosphorylation of NPR2. **(B)** Graph showing the results of 4 experiments like that shown in **A** (mean ± s.e.m.). **(C)** Membranes from follicles treated with or without okadaic acid followed by LH, as described above, were assayed for NPR2 guanylyl cyclase activity (4 experiments). Values not indicated by the same letter are significantly different. We also attempted to determine the effect of okadaic acid on the LH-induced decrease in cGMP, but these experiments were not interpretable because okadaic acid alone caused cGMP to decrease in some of the trials.

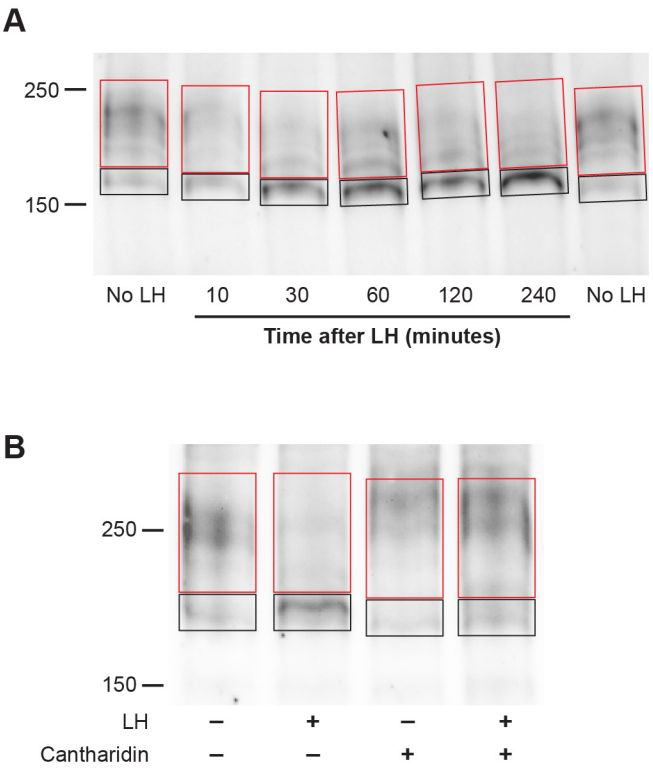

Figure S3. Blot images for figures 3A and 4A, with red boxes indicating the upper region (more phosphorylated) and lower band (less phosphorylated) for which immunostaining intensity was measured. (A) Figure 3A. **(B)** Figure 4A.

SUPPLEMENTARY MATERIALS AND METHODS

Isolation and culture of rat ovarian follicles

Preovulatory follicles, 700 - 900 μm in diameter, were dissected from the ovaries of rats that had been injected 48 hours previously with equine chorionic gonadotropin. Approximately 30 follicles were obtained per rat. The follicles were placed on Millicell culture inserts (PICMORG50, Millipore, Billerica, MA; 10-30 follicles per insert). MEMα medium (Invitrogen, Carlsbad, CA) was supplemented with 25 mM $NaHCO_3$, 3 mg/ml BSA, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 50 μg/ml streptomycin, and 75 μg/ml penicillin G. The follicles were cultured at 37°C in 5% CO_2 in air, and experimental procedures were started one to 4 hours after isolation. LH and other reagents were applied to the medium under the culture membrane, and 200-400 μl was also added to the top of the membrane to ensure rapid exposure of the follicles. Ovine LH and equine chorionic gonadotropin, purified from biological sources, were obtained from A.F. Parlow (National Hormone and Peptide Program, Torrance, CA). LH was used at a saturating concentration (10 μg/ml; approximately 350 nM). The kinetics of nuclear envelope breakdown in response to LH were determined by incubating isolated follicles with LH, and at various times afterwards, opening them with 30 gauge needles to release the cumulus-oocyte complex for observation of the presence or absence of the nucleus and nucleolus.

Preparation of crude membranes from rat follicles

To prepare crude membranes from rat follicles, 20-100 follicles were washed in PBS and then lysed in phosphatase inhibitor buffer (buffer A) containing 25 mM Hepes (pH 7.4), 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 20% glycerol, 1 μM microcystin-LR (Cayman Chemical Co., Ann Arbor, MI), and protease inhibitors (complete Mini, EDTA-free; Roche Applied Science), in a 0.1 ml glass homogenizer (Wheaton, Millville, NJ) on ice. The follicle wash procedure was done at room temperature and was started 3 minutes before the homogenizer was placed on ice. LH incubation times refer to the times at which the homogenizer was chilled. The homogenate (200-400 μl volume) was centrifuged at 10,000xg for 20

minutes at 4°C; the pellet was resuspended in buffer A using a probe sonicator. Protein content was determined by a BCA assay (Thermo Fisher Scientific, Rockford, IL). The crude membrane fraction contained approximately 10 μg of protein per follicle. Aliquots were frozen in liquid N_2 and stored at -80°C. Crude membranes were also prepared from HEK-293T cells stably expressing NPR2, from plates of cells at 70-80% confluency that had been serum starved for >2 hours.

Measurement of guanylyl cyclase activity in follicle membranes

Guanylyl cyclase assays were conducted as previously described (Robinson and Potter, 2011). Assays were performed at 37°C using 3-20 μg of follicle protein per assay tube, in the presence or absence of CNP (1 μM except as indicated). The reaction mixture contained 25 mM Hepes (pH 7.4), 50 mM NaCl, 0.1% BSA, 1 mM EDTA, 0.5 μM microcystin, 5 mM $MgCl_2$, as well as 1 mM of the allosteric activator, ATP, and 1 mM of the substrate, GTP. 5 mM creatine phosphate and 0.1 mg/ml creatine kinase were included in the reaction mixture to maintain ATP and GTP concentrations. 0.5 mM isobutylmethylxanthine was included to inhibit cGMP phosphodiesterase activity.

Immunoprecipitation of NPR2 from rat follicle membranes

Crude membrane samples (130-230 μg protein) were diluted to 400 μl in immunoprecipitation buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 50 mM NaF, 10 mM NaH_2PO_4, 2 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 μM microcystin-LR, and protease inhibitors. NPR2 was immunoprecipitated by incubation with 0.6 μl of 6328 rabbit polyclonal antiserum made against the 10 C-terminal amino acids of NPR2 (Abbey and Potter, 2002) for one hour at 4°C, and then with 25 μl of washed protein AG magnetic beads (Thermo Fisher Scientific, Rockford, IL) overnight at 4°C. The beads were washed and resuspended in Laemmli sample buffer with 75 mM dithiothreitol. Protein was eluted by heating at 70°C for 10 minutes. Approximately 50% of the membrane NPR2 was recovered after immunoprecipitation.

Phos-tag acrylamide gel electrophoresis and Western blotting

Phosphorylated forms of NPR2 or PDE5 were separated by electrophoresis on SDS-PAGE gels made with 6% acrylamide copolymerized with 25 μM Phos-tag-acrylamide (WAKO Chemicals USA, Richmond, VA) and 100 μM $MnCl_2$ (145 x 160 x 1.5 mm gel dimensions). Gels containing 6% acrylamide, but without Phos-tag-acrylamide or $MnCl_2$, were used for comparison. Immunoprecipitated NPR2, or lysates of follicles that had been washed in PBS and then sonicated in Laemmli sample buffer with 75 mM dithiothreitol, were electrophoresed at 25 mA/gel for 6-8 hours at 4°C. The gels were then incubated for 20 minutes in 400 ml transfer buffer (100 mM Tris, 192 mM glycine, no SDS or methanol) containing 1 mM EDTA to chelate Mn^{2+}, and then washed for 20 minutes in 400 ml transfer buffer alone to remove Mn^{2+}-EDTA.

Protein was transferred to a nitrocellulose membrane for 17-20 hours with 500 mA constant current at 4°C. The membrane was stained with Ponceau-S, and blocked with 0.1% Tween and 2% milk. Blots for NPR2 were probed overnight at 4°C with a 1:50,000 dilution of the 6328 antiserum (see main text), and then with a 1:500 dilution of Clean-Blot IP Detection Reagent coupled to HRP (Thermo Fisher Scientific, Rockford, IL). Blots for PDE5 were probed overnight with a 1:500 dilution of the 2395 antibody from Cell Signaling Technology (see main text) and then with a 1:5000 dilution of a goat-anti-rabbit antibody coupled to HRP (catalog # sc-2004; Santa Cruz Biotechnology Inc., Dallas, TX). Blots were developped using ECL Prime (GE Healthcare Bio-Sciences, Piscataway, NJ), and imaged using a charge-coupled device camera (G:Box Chemi XT4, Syngene, Frederick, MD).

In vitro dephosphorylation of NPR2 in follicle membranes

To confirm that the LH-induced acceleration of NPR2 migration in a Phos-tag gel was due to dephosphorylation (Fig. 2E,F), follicle membranes were incubated at 30°C for 30 min, either with phosphatase inhibitors (50 mM NaF + 2 mM EDTA + 1 μM microcystin-LR), or under conditions that

promoted phosphatase activity (no phosphatase inhibitors, and 2 mM $MgCl_2$; see Bryan and Potter, 2002). NPR2 was then immunoprecipitated and separated on a gel containing Phos-tag acrylamide; NPR2 was visualized by western blotting. To prepare membranes for these assays, follicles were homogenized in buffer A without microcystin-LR, to avoid irreversible modification of phosphatases. After centrifugation, the membranes were suspended in a buffer containing 25 mM Hepes, 50 mM NaCl, 20% glycerol, and protease inhibitors. After incubation under the indicated conditions, aliquots were frozen for immunoprecipitation and western blotting (Fig. 2 E,F), and for guanylyl cyclase assays (Fig. 2G).

Bryan, P. M. and Potter, L. R. (2002). The atrial natriuretic peptide receptor (NPR-A/GC-A) is dephosphorylated by distinct microcystin-sensitive and magnesium-dependent protein phosphatases. *J. Biol. Chem.* **277**, 16041-16047.

In vitro phosphorylation of PDE5 in follicle lysates

To confirm that the LH-induced retardation of PDE5 migration in a Phos-tag gel was due to phosphorylation (Fig. 5D), a lysate of follicles was incubated with the catalytic subunit of protein kinase A (PKA_c, kindly provided by Jackie Corbin, Vanderbilt University), following the procedure described by Rybalkin et al., 2002. The follicles were lysed in a glass homogenizer in a buffer containing 50 mM Tris, pH 7.5, 1.5 mM EDTA, 25 mM NaF, 0.2 mM Na vanadate, and protease inhibitors (Roche complete Mini) followed by sonication. Aliquots containing 100 μg protein were then incubated at 30°C for 30 minutes with 4 μM PKA_c, or with the buffer in which the PKA_c was dissolved. Reactions were performed with or without 10 μM cGMP, which is required for phosphorylation of PDE5 by PKA_c (Corbin et al., 2000). The samples were then spin-dialyzed into a buffer compatible with Phos-tag gel electrophoresis (50 mM Tris, pH 7.5, 25 mM NaF, 0.2 mM Na vanadate, and protease inhibitors), using a 0.5 ml, 10K Amicon Ultra centrifugal filter (EMD Millipore, Billerica, MA).