Supplementary data

Protein Kinase B/Akt Phosphorylation of PDE3A and Its Role in Mammalian Oocyte Maturation

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Supplementary materials and methods

Materials

[^{3}H]cAMP and [γ-^{32}P]ATP were obtained from New England Nuclear Science Inc. (Boston, MA). AG 1-X8 resin was from Bio-Rad Laboratories (Hercules, CA) and ECL Western blot detention kit was from Amersham-Pharmacia Biotech (Piscataway, NJ). DMEM and fetal bovine serum were purchased from Invitrogen Corp. (Carlsbad, CA). Immobilon was from Millipore Corp. (Bedford, MA). Unless otherwise noted, all chemicals were the purest grade available from Sigma-Aldrich Chemicals (St. Louis, MO).

Cell culture and transfection

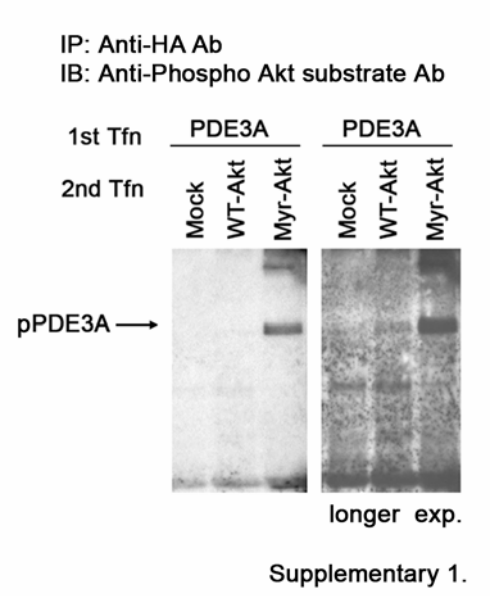
For recombinant expression of PDE constructs, Hek293 or MA10 were grown to 70% confluence on 100-mm dishes and then transfected using Transfectamine 2000^{TM} transfection system (Invitrogen Corp.) according to the manufacturer's protocol. After 10 h of further incubation, cells were divided into 60-mm dishes and incubated again for 6 h. Cultures were then transfected with second DNA constructs and incubated an additional 19 h. Cells were then harvested in a hypotonic buffer containing 10 mM Tris-HCl (pH 7.5), 0.01% NP40, 2 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM β-mercaptoethanol, 1 μM microcystin-LR, 4 μg/ml aprotinin, 0.7 μg/ml pepstatin, 0.2 mM PMSF, and 0.5 μg/ml leupeptin. The cells were incubated at 4 °C with rotation for 15 min then centrifuged at 1.6 x 10,000 *g* for 10 min. The membrane fractions were collected and resuspended in same buffer with 0.05% of

NP40. After 15 min with rotation at 4 °C, the membrane debris was spun down at 1.6 x 10,000 *g* for 10 min and supernatants were collected for PDE assay and Western blot.

In vitro *mRNA synthesis and injection into* Xenopus *oocytes*

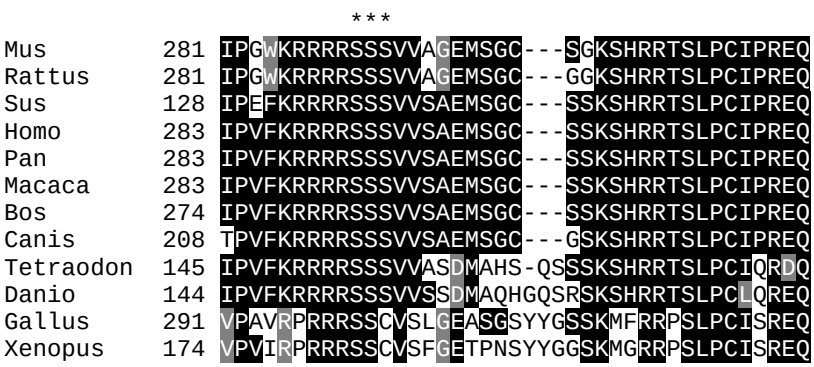
To express PDE3A in *Xenopus* oocytes, pcDNA3.1- PDE3A constructs were transcribed using T7 polymerase with mMessage mMachine™ and adding the polyA tail using poly(A) tailing kit ™ according to manufacturer's protocol (Ambion Inc., Austin, TX). Dumont Stage VI oocytes were selected for all experiments. Oocyte storage and experiments were carried out in OR2 solution (5 mM HEPES (pH 7.8), 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4). The mRNAs or H_2O (vehicle) were injected using a micromanipulator (Narishige USA Inc., Long Island, NY) into defolliculated *Xenopus* oocytes. Oocyte maturation was induced by stimulation with 500 nM progesterone or by injection with indicated concentration of *pde3a* mRNA and treatment with 1 μM insulin. Resumption of meiosis was scored by the appearance of a white spot on the animal pole of the oocyte. At the indicated hours after these treatments, oocytes were harvested in 3 μl of Tris buffer (250 mM sucrose, 10 mM Tris-HCl (pH 8.0), 0.01% NP40, 0.1 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 10 mM NaF, 10 mM sodium pyrophosphate, 5 mM β-mercaptoethanol, 1 μM microcystin, 4 μg/ml aprotinin, 0.7 μg/ml pepstatin, 0.2 mM PMSF, and 0.5 μg/ml leupeptin) per oocyte. The equivalents of 15 oocytes of lysate were used for PDE assay and lysates from 10 oocytes were assayed by Western blotting for protein expression.

Supplementary data



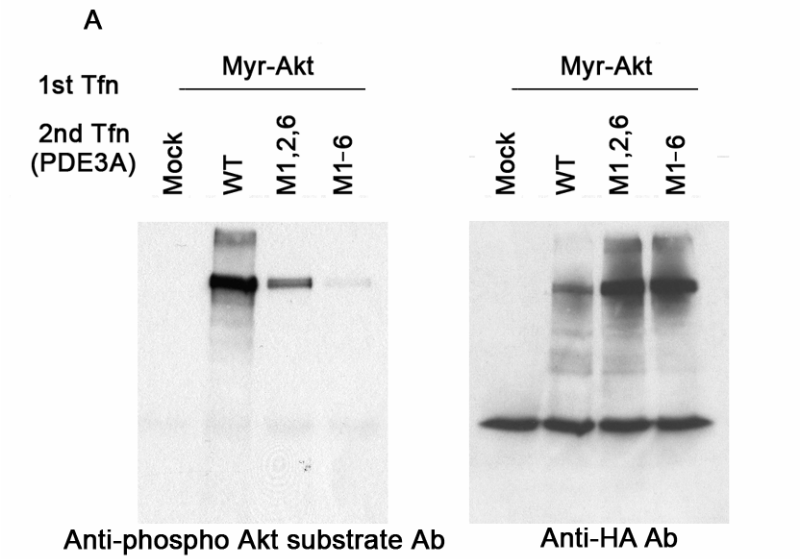
Supp. Figure 1. PDE3A is phosphorylated by coexpression with Akt.

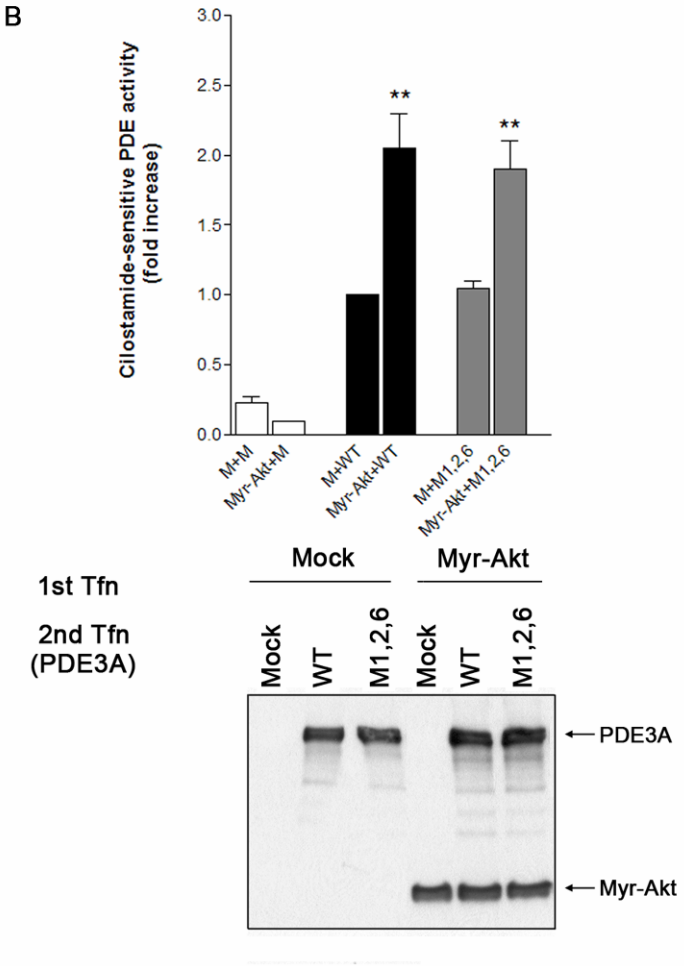
PDE3A transfected (PDE3A) cells were cotransfected with Mock, wild type (WT)-Akt and myr-Akt. After immunoprecipitation with HA antibody, SDS-PAGE was carried out. After transfer to the PVDF membrane, the blot was incubated with anti-phospho Akt substrate antibodies. An increase in the phosphorylation of PDE3A was detected in the coexpression with WT-Akt after longer exposure (right panel) as well as myr-Akt.



Supplementary 2.

Supp. Figure 2. Alignment of amino acid sequence of PDE3A from different species. Sequence alignment was performed using MUSCLE (http://www.drive5.com/muscle/) software. Residues identical among two sequences are boxed in black. Serine residues marked with asterisk indicate the potential PKB/Akt phosphorylation sites. The putative PKB/Akt phosphorylation site (Serine 290 in the mouse) is conserved in all species analyzed. No homology in the amino terminus of the *Strongylocentrotus purpuratus* sequence (XP_793920) could be found. Accession number for the sequences are: *Mus musculus* (NP_061249.1), *Rattus norvegicus* (NP_059033.1), *Sus scrofa* (NP_998901.1), *Homo sapiens* (AAB18673.1), *Pan troglodytes* (XP_520783.2), *Macaca mulatta* (XP_001096963.1), *Bos taurus* (XP_609268.2), *Canis familiaris* (XP_853486.1), *Tetraodon nigroviridis* (CAF87968.1), *Xenopus laevis* (AAH87549), *Danio rerio* (CAI21166), *Gallus gallus* (NP_001026353)

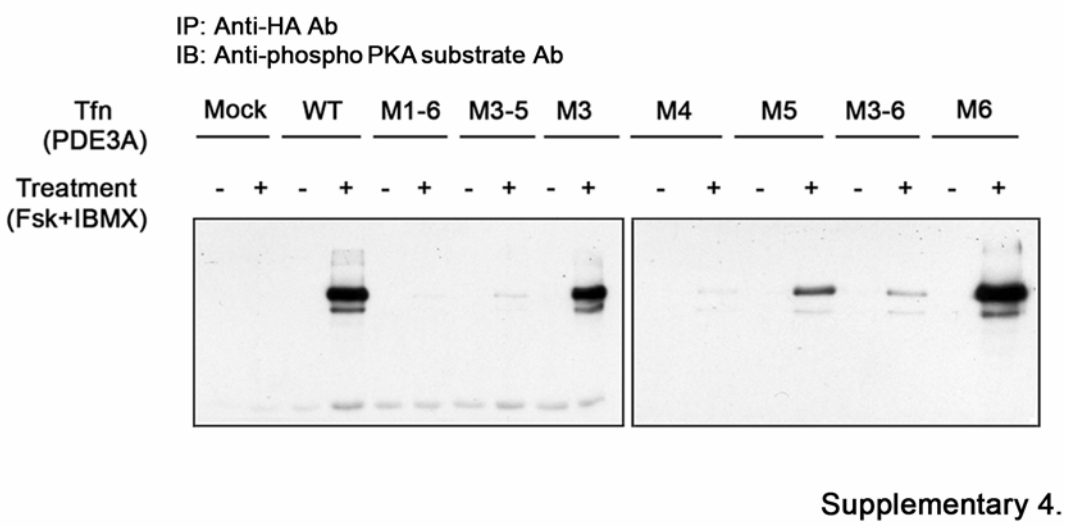




Supplementary 3.

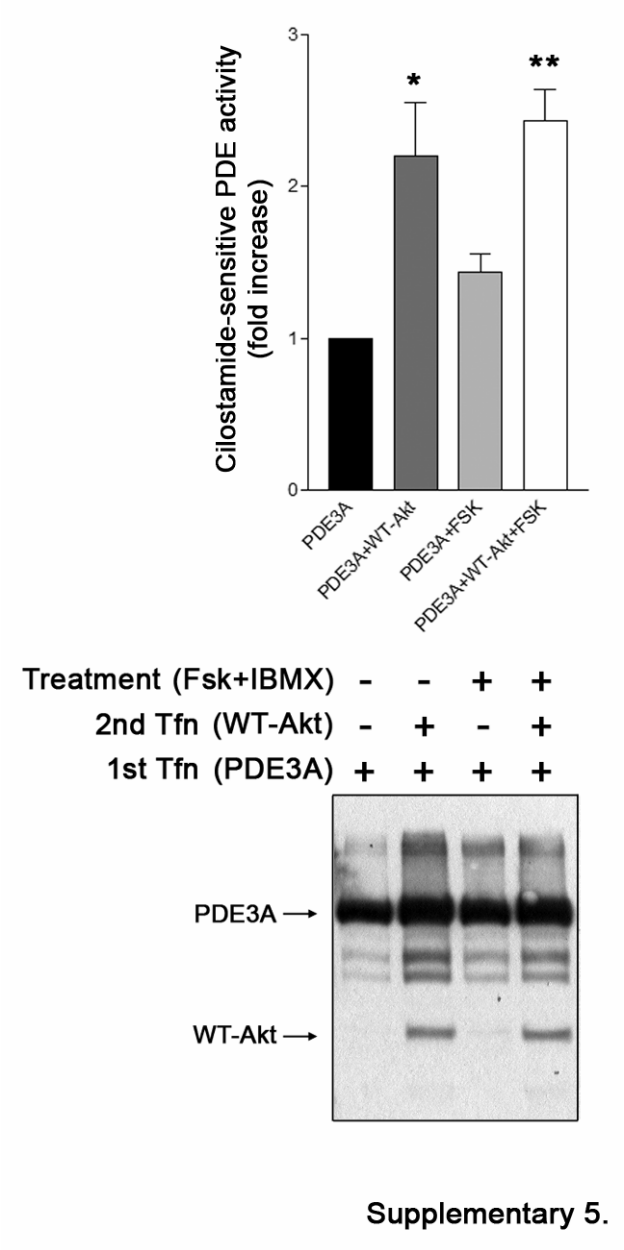
Supp. Figure 3. PKB/Akt-induced PDE3A activation is depend on the phosphorylation on S290-292 serine residues in PDE3A.

A, After cotransfection with myr-Akt and immunoprecipitation, the phosphorylation state of the M1,2,6 mutant PDE3A-HA was determined using anti-phospho Akt substrate antibody. The immunoprecipitated mutant PDE3A was phosphorylated less than wild type but more than M1-6 mutant (left panel). This decrease of phosphorylation is due to the removal of the additional putative PKB phosphorylation sites. The amount of immunoprecipitated protein was comparable as determined by anti-HA antibodies (right panel). *B*, The cilostamide-sensitive PDE activity (upper panel) was measured after serial transfection of Myr-Akt or empty vector (1st Tfn) and wild type or M1,2,6 PDE3A mutants (2nd Tfn). The increase of activity by myr-Akt in the M1,2,6 mutant is comparable to that of the wild type enzyme. The amount of proteins expressed were comparable as determined by anti-HA antibodies on cell lysate (lower panel). ** indicates $P<0.01$ versus mock control.



Supp. Figure 4. PKA phosphorylates mainly serine 291 of PDE3A.

After expression of the different construct, treatment with 10 μM forskolin and 1 mM IBMX for 30 min, and immunoprecipitation of the extracts, the phosphorylation states of the WT, M3, M4, M5, M6, M3-5, M3-6, or M1-6 mutant PDE3A-HAs were determined using anti-phospho PKA substrate antibody (Cell Signaling #9624). Phosphorylation of PDE3A is not detected upon mutation of M4 (S291) site.



Supp. Figure 5. PDE3A activity is increased by PKA.

Cells were transfected with PDE3A (1st Tfn) and wild type Akt (2nd Tfn). After treatment with 10μM forskolin, and 1mM IBMX for 30 min to activated PKA, cilostamide-sensitive PDE activity was measured in cell lysates (upper panel). The Akt and PKA both activate PDE3A activity; however, there is no synergic effect on the PDE3A activity when the cells were treated with forskolin and IBMX. ** represents $P<0.01$ and * $P<0.05$ compared to non treated control. The expression levels of PDE3A and Akt were confirmed with HA antibodies (lower panel; PDE3A, WT-Akt).