Phosphodiesterases in the rat ovary: effect of cAMP in primordial follicles

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Abstract

Phosphodiesterases (PDEs) are important regulators of the intracellular cAMP concentration, which is a central second messenger that affects a multitude of intracellular functions. In the ovaries, cAMP exerts diverse functions, including regulation of ovulation and it has been suggested that augmented cAMP levels stimulate primordial follicle growth. The present study examined the gene expression, enzyme activity and immunolocalization of the different cAMP hydrolysing PDEs families in the rat ovary. Further, the effect of PDE4 inhibition on primordial follicle activation in cultured neonatal rat ovaries was also evaluated. We found varied expression of all eight families in the ovary with *Pde7b* and *Pde8a* having the highest expression each accounting for more than 20% of the total PDE mRNA. PDE4 accounted for 15–26% of the total PDE activity. Immunoreactive PDE11A was found in the oocytes and PDE2A in the corpora lutea. Incubating neonatal rat ovaries with PDE4 inhibitors did not increase primordial follicle activation or change the expression of the developing follicle markers *Gdf9*, *Amh*, *Inha*, the proliferation marker *Mki67* or the primordial follicle marker *Tmeff2*. In addition, the cAMP analogue 8-bromo-cAMP did not increase AKT1 or FOXO3A phosphorylation associated with follicle activation or increase the expression of *Kitlg* known to be associated with follicle differentiation but did increase the *Tmeff2*, *Mki67* and *Inha* expression in a dose-dependent manner. In conclusion, this study shows that both *Pde7b* and *Pde8a* are highly expressed in the rodent ovary and that PDE4 inhibition does not cause an increase in primordial follicle activation.

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Introduction

cAMP is a fundamental intracellular second messenger involved in signal transduction for many hormones, growth factors and neurotransmitters (Zaccolo 2011). In the ovaries, cAMP is of utmost importance being instrumental as a second messenger for the follicle stimulating hormone (FSH) and luteinising hormone (LH) receptors, and is central in growth and development of follicles in all stages (Conti 2002, Zhang *et al.* 2004, Menon & Menon 2012).

Inactivation of cAMP is carried out by phosphodiesterases (PDEs), which exists as 11 families of enzymes each with one to four members. Each family differs in regulation and specificity for cAMP and cGMP. PDE1–4, PDE7–8 and PDE10–11 all catalyse hydrolysis of cAMP, while the remaining three PDE families solely hydrolyse cGMP (Azevedo *et al.* 2014). In the rodent ovary, PDE4D and PDE3 are known to exert important roles in regulation of oocyte maturation and ovulation (Tsafiriri *et al.* 1996, Park *et al.* 2003, Conti *et al.* 2012). In addition, the presence of PDE8 has been reported in the

bovine and human ovary (Sasseville *et al.* 2009, Petersen *et al.* 2015) but has not been studied in rodents.

Elevated levels of cAMP have also been shown to promote folliculogenesis and follicle survival in cultured human ovarian cortex stimulated with the cAMP analogue 8-bromo-cAMP (Zhang *et al.* 2004). The exact mechanism responsible for primordial follicle activation to enter growth is uncertain but recent studies have shown that the PI3Kinase/AKT1/mTORC1/FOXO3A pathway is involved (Oktem & Urman 2010). Activation of this pathway leads to phosphorylation of both AKT1 (RAC-alpha serine/threonine-protein kinase) and its downstream target FOXO3A (Forkhead box protein O3). The latter is a transcription factor that upon phosphorylation is exported from the nucleus allowing transcription and follicle activation to take place. This occurs within 6 h after activation of the pathway (Li *et al.* 2010). It is possible that cAMP interacts with the PI3Kinase pathway through exchange protein directly activated by cAMP (EPAC) in the oocyte as seen in skeletal muscle (Baviera *et al.* 2010). Alternatively, cAMP may increase the production of Kit ligand (KITLG),

also known as stem cell factor, in the granulosa cells (GC) as gene expression of KITLG has been shown to be stimulated by cAMP in Sertoli cells, and in ovarian cancer cells (Shaw *et al.* 2002, Grimaldi *et al.* 2003). KITLG can also activate AKT through its receptor cKIT located on the oocyte (De Miguel *et al.* 2002).

In addition to the effects in the reproductive system, PDEs exert functions in many other cells located in a number of organs. PDE inhibitors have attracted a great deal of attention as possible drug candidates for treatment of many diseases such as inflammatory and neurological diseases. However, the majority of PDE inhibitors have failed to reach the market due to adverse effects (Gavalda & Roberts 2013, Azevedo *et al.* 2014). Inhibition of PDE4 is known to affect ovulation and the activity of corpora lutea (CL) in rodents (Park *et al.* 2003, McKenna *et al.* 2005, Losco *et al.* 2010) but a possible stimulatory effect on the activation of primordial follicle growth mediated by cAMP would be regarded as a serious side effect due to its irreversible nature. The effects on the ovary by PDE4 inhibitors are most likely caused by elevated cAMP, but the presence of other cAMP hydrolysing PDEs in the follicle may alter the effect of specific PDE4 inhibitors and limited information is available about other members of PDEs in the rodent ovary.

The aim of the present study was to provide basic information on the presence, expression and immunolocalization of all cAMP hydrolysing PDEs during folliculogenesis in rats and to study the effect of PDE4 inhibition on primordial follicle activation in an *in vitro* neonatal rat model.

Materials and Methods

Animals

Ovaries were isolated from day 4 post-natal (PN) Han-wistar rats (Harlan, Horst, The Netherlands) as previously published (Petersen & Andersen 2014). For isolation of GC, 10-week-old Wistar rats (Taconic, Ejby, Denmark) were stimulated with 22.5 IU FSH/LH (Menopur, Ferring, Copenhagen, Denmark) and two groups were injected after 46 h with 20 IU human chorionic gonadotropin (hCG) (Pregnyl, MSD, Ballerup, Denmark). After 2 h or 4 h, the animals were sacrificed and the ovaries removed. The third group acting as control did not receive hCG and was sacrificed after 48 h. Individual follicles were punctured and the GC were isolated with a Pasteur pipette and washed repeatedly to remove blood contamination. The cells were snap-frozen and stored at -80°C for later RNA isolation or PDE activity measurement.

Follicles from Han-Wistar rats PN day 8–10 and CL from mature rats were isolated enzymatically by incubating the ovaries in MEM alpha media (Life Technologies) supplemented with 0.1% w/v Collagenase (Sigma–Aldrich) and 0.8% w/v DNase (Sigma–Aldrich) for 20 min at 37°C . The ovaries were transferred to PBS containing 5% v/v foetal bovine serum (FBS) (Life Technologies), incubated for 10 min

and transferred to fresh culture media where the follicles and CL were dissected free with a pair of 30G hypodermic needles. Sequential filtering of the media through a 40 and 11 μm nylon filter (Millipore, Hellerup, Denmark) was used to separate follicles into two groups of 11–40 and 40–300 μm in diameter, a method previously used with primate ovaries (Hornick *et al.* 2012).

The Danish Animal Experiments Inspectorate (Dyreforsøgstilsynet) approved the study.

Culture

The ovaries were cultured in Nunc four well dishes (Fisher Scientific, Hvidovre, Denmark) on 12 mm floating hydrophilic PTFE cell culture insert with pore size of 0.4 μm (Millipore) covered with a small drop of media. The culture media consisted of MEM alpha, 1% v/v human serum albumin (CSL Behring, Lyngby, Denmark), 1% v/v insulin–transferrin–selenium (Life Technologies), 1% v/v Pen Strep (Life Technologies), 1% v/v glutamax (Life Technologies) and 1% v/v FBS, 25 IU/l FSH (Puregon, MSD, Ballerup, Denmark). Each well contained 200 μl medium and the media was changed every other day. As selective PDE4 inhibitors, the two substances LEO29102 and its pyridine N-oxide LEO28386 (LEO Pharma, Ballerup, Denmark) were used (Felding *et al.* 2014). The inhibitors were dissolved in DMSO (Sigma–Aldrich) and diluted in culture media until the final DMSO concentration was 0.01%. For experiments with the cAMP analogue 8-bromo-cAMP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), DMSO was replaced with water. The ovaries were removed after culturing for 6 h for immunohistochemistry, 2 h for western blotting and 7 days for qPCR and follicle classification. Briefly, the ovaries were washed in PBS and snap-frozen in liquid nitrogen or embedded in 2% agarose and fixed in Bouin's solution.

RNA extraction and RT-qPCR

RNA extractions were performed with the Dynabeads mRNA DIRECT kit (Life Technologies) according to the manufacturer's instructions with the addition of 1% v/v protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma–Aldrich) to the lysis buffer. 200 μl lysis buffer and washing buffer were used and the mRNA was eluted in 25 μl elution buffer. RT was carried out using the High capacity cDNA kit (Life Technologies). Real-time quantitative PCR was performed as described previously (Petersen & Andersen 2014) with TaqMan primer probe assays (Assay ID) against *Pde1b* (Rn00575591_m1), *Pde1c* (Rn00579334_m1), *Pde2a* (Rn01648917_m1), *Pde3a* (Rn00569192_m1), *Pde3b* (Rn00568191_m1), *Pde4a* (Rn00565354_m1), *Pde4b* (Rn00566785_m1), *Pde4c* (Rn01754406_m1), *Pde4d* (Rn00566798_m1), *Pde7a* (Rn01493469_m1), *Pde7b* (Rn00590117_m1), *Pde8a* (Rn01648408_m1), *Pde8b* (Rn01489992_m1), *Pde10a* (Rn00673152_m1), *Pde11a* (Rn01639097_m1), *Gdf9* (Rn00572328_m1), *Kitlg* (Rn01502851_m1), *Inha* (Rn00561423_m1), *Tmeff2* (Rn02110772_s1), *Amh* (Rn00563731_g1), *Rpl32* (Rn00820748_g1), *Mki67* (Rn01451446_m1) and *Gapdh* (Rn01775763_g1). The expression of

Pde1b-Pde11a was normalised to the total amount of PDE mRNA according to the following equation:

$$\text{Proportion}_{Pde_i} = \frac{2^{-Cq_{Pde_i}}}{\sum_j 2^{-Cq_{Pde_j}}}, \quad i, j \in \{1b, 1c, 2a, \dots, 8b, 10a, 11a\}.$$

The amplification efficiency for the TaqMan assays according to the manufacture is 100% (<http://docs.appliedbiosystems.com/pebi/docs/00113186.pdf>). This was validated by linear regression with *Gapdh* as the reference gene (Cui *et al.* 2015) with adjustment for sample group (included as an additional independent variable). The expression of the remaining genes is presented as $2^{-\Delta Cq}$ normalized to Rpl32 (Schmittgen & Livak 2008).

PDE activity assay

GC were lysed in 50 mM Tris-HCl (pH 7.5) containing 0.05% Triton-X 100, 1.7 mM EGTA, 8.3 mM MgCl₂, 6 mM CaCl₂ and 1% of protease inhibitor cocktail for mammalian cells (Sigma-Aldrich). Aliquots of the lysate were assayed for total PDE activity as well as roflumilast insensitive (total non-PDE4) activity in the absence or presence of 0.5 µM roflumilast (Santa Cruz Biotechnologies). The roflumilast sensitive PDE4 activity was determined by subtracting the roflumilast-insensitive activity from the total activity. The PDE assays were performed using a cAMP-PDE SPA assay as detailed previously (Petersen & Andersen 2014) with the following modifications: 0.1% BSA (Sigma-Aldrich) was used in the reaction buffer and the activity was measured once after 15 min.

Western blot

Ovaries and rat tongue were homogenized using cryogrinding and lysed in RIPA buffer with 1% v/v protease inhibitor cocktail and 1% v/v phosphatase inhibitors cocktail 3 (Sigma-Aldrich). Protein concentration was determined using the Bio-Rad DC Protein assay (Bio-Rad). Total protein of 40 µg for PN day 8 ovaries and tongue, and 13 µg for PN day 6 ovaries were loaded on a Bis-Tris 4–12% Gel and electrophoretic separated. The proteins were transferred to an Invitrolon PVDF membrane (Life Technologies) and equal protein load was checked using CPTS stain (Bickar & Reid 1992) followed by destaining in a 5% w/v BSA (Sigma-Aldrich) solution in Tris Buffed Saline and drying of the membrane after an ethanol wash. Western blotting was carried out as described previously (Naryzhny 2009) using rabbit antibodies raised against FOXO3A (AbCam, Cambridge, UK), phospho-FOXO3A (AbCam), phospho-AKT1 S473 (AbCam) and peroxidase labelled donkey anti-rabbit secondary antibody (Agrisera, Vännas, Sweden). The blot was developed with SuperSignal West Femto Substrate (Thermo Fisher Scientific, Hvidovre, Denmark) and imaged with a Canon EOS 1100D camera (Canon, Tokyo, Japan) cooled with dry ice.

Staining and follicle classification

The fixated ovaries were dehydrated and embedded in paraffin, cut in 10 µm serial sections and stained with haematoxylin

and PAS. The slides were digitalized using a Panoramic MIDI slide scanner (3DHISTEC, Budapest, Hungary) equipped with a Plan-Apochromat 20×/0.8 objective (Carl Zeiss, Jena, Germany) and the sections were isolated and aligned using custom software. The follicles were classified based on the

Figure 1 Gene expression of cAMP hydrolysing PDEs in the rat ovary evaluated by RT qPCR. Follicles were isolated from rats PN day 8–11. Corpora lutea from mature rats and granulosa cells from FSH+LH stimulated adult rats at various timepoints after hCG stimulation. The expression was normalized to the total PDE mRNA amount. Different letters indicate statistical significant difference. Error bars indicate S.E.M.

Table 1 Total and type 4 cAMP phosphodiesterase activity in rat granulosa cells before and after hCG stimulation.

	Total PDE activity (pmol/min per mg total protein)	PDE4 activity (pmol/min per mg total protein)
Pre-hCG	42.0±7.2	6.5±1.9
2 h post-hCG	39.7±2.5	9.1±3.7
4 h post-hCG	37.6±2.9	9.9±2.2

Mean ± S.E.M., $n=5$.

appearance of the follicles according to Lintern-Moore *et al.* (1974) into five categories: primordial with flatten GC (class B), intermediary with at least one cuboid GC (class B/C), primary with all GC being cuboidal (class C), secondary with more than one layer of GC (class D1) and antral with visible antrum (class D2-F). Follicle classification and area measurement were performed on oocytes with visible nucleoli on every fifth section using TrakEM2 (Cardona *et al.* 2012).

Immunohistochemistry analysis

For IHC detection of PDEs, sections were deparaffinised with xylene and rehydrated through ethanol series. Antigen retrieval was performed in citrate buffer, pH 6 (Dako, Glostrup, Denmark) for PDE3A and PDE4D. The slides were blocked in an antibody dilution buffer (Dako) followed by incubation of primary antibodies diluted in antibody blocking buffer overnight at 4 °C. The slides were washed and detected using Dako envision and DAB+. Haematoxylin was used for nuclear counter stain. The primary antibodies raised in rabbits against PDE1B, PDE1C, PDE2A, PDE3A, PDE3B, PDE4A, PDE4B, PDE4C, PDE4D, PDE7A, PDE7B, PDE8A, PDE8B and PDE11A were all purchased from FabGennix (Frisco, TX, USA). The images were captured using a Panoramic MIDI Slide scanner. For detection of FOXO3A, no blocking was used, antigen retrieval was performed with Tris-EDTA pH 9 and the washing buffer used was water with 0.5% v/v Tween-20 (Merck). Anti-FOXO3A (AbCam) was used as the primary antibody and peroxidase labeled donkey anti-rabbit as secondary antibody. DAB+ (Dako) was used for detection. Images were captured

with a CANON EOS 1100D camera mounted on an Olympus IX-70 microscope (Olympus) equipped with an Olympus UAPO N 340 40×/1.15 Water immersion objective.

Statistical analysis

ANOVA was used for comparing differences in gene expression with adjustment for multiple comparisons using the Benjamini–Hochberg correction and with a logit transformation applied to the relative PDE mRNA gene expression (Warton & Hui 2011). χ^2 was used for comparing differences in follicle class distribution. All values are expressed as mean ± S.E.M. A P value <0.05 was considered to be statistical significant.

Results

qPCR and PDE activity

In the neonatal ovary, *Pde8a* was the highest expressed PDE, followed by *Pde3a*, *Pde7b* and *Pde7a* (Fig. 1). The remaining PDEs each accounted for <10% of the total PDE mRNA. *Pde4a*, *Pde4b*, *Pde4c*, *Pde8b* and *Pde11a* had a very low expression in total contribution, <1%. The smaller follicles 11–40 µm diameters followed the same pattern although *Pde3a* was the highest expressed PDE followed by *Pde8a*. In the larger follicles, *Pde8a* had the highest expression followed by *Pde7b*, *Pde3a* and *Pde7a*, although there were no statistical significant differences between the two follicle groups.

In the GCs, only *Pde8a* and *Pde7b* each accounted for more than 10% of the total PDE mRNA. Only *Pde10a* was affected by hCG increasing 4.4-fold after 4 h post-hCG. In contrast to the whole ovary and follicles, the expressions of both *Pde1b*, *Pde2a*, and *Pde3b* were below 1%. The CL had the highest expression of *Pde8a* accounting for 60% followed by *Pde2a* at 12%. The remaining PDEs each accounted for <10% of the total PDE mRNA.

Table 2 Immunohistochemical staining for phosphodiesterases in different types of follicles and in the corpus luteum of the rat ovary.

Antibody raised against	Primordial	Primary	Secondary	Antral	Corpus luteum
PDE1B	+	+	+	–	++
PDE1C	+	+	+	+	++
PDE2A	–	–	–	–	+
PDE3A	++ ^a	++ ^a	++ ^a	+	+
PDE3B	+ ^a	+ ^a	+ ^a	++ ^b	+
PDE4A	–	–	+ ^a	+ ^a	++
PDE4B	+	+	+	+	+
PDE4C	++ ^a	++ ^a	++ ^a	++ ^a	+
PDE4D	+	+	+	+	+
PDE7A	+ ^a	+ ^a	+ ^a	+ ^a	+
PDE7B	+ ^a	+ ^a	+ ^a	+ ^a	+
PDE8A	++	++	++	++	+
PDE8B	–	–	–	++ ^b	+
PDE11A	+++ ^a	+++ ^a	++ ^a	++ ^a	+

Staining intensity: + + +, strong staining; + +, moderate staining; +, weak staining; –, no staining.

^aOocyte localization. ^bTheca cells.

Figure 2 Immunohistological staining for selected PDEs in adult rat ovary. PDE2A was present in the corpora lutea (A), PDE3A in follicles and corpora lutea (B), PDE3B showed string staining in the oolemma (C), PDE4D was present in follicles and corpora lutea (D), PDE7B stained faintly in the corpora lutea and follicles (E), PDE8A stained both follicles and corpora lutea (F), PDE11A stained primordial follicles strongly (G), the control without primary antibody stained the follicle fluid faintly (H). Scale bar 1 mm.

In all groups, the expression of *Pde4a*, *Pde4b* and *Pde4c* were very low. *Pde4d* accounted for 0.5–3.2%.

In contrast, the enzymatic activity of PDE4 accounted for 15–26% of the total PDE activity in the GCs with a tendency to a higher activity post-hCG (Table 1).

IHC

The staining patterns for various PDEs are summarized in Table 2. PDE2A was present in the CL (Fig. 2A). PDE3A was detected in the oocytes and with lower intensity in the GC (Fig. 2B). PDE3B stained strongly in the oolemma (Fig. 2C). PDE4D was present in follicles and CL (Fig. 2D). Positive staining of PDE7B was seen in the oocytes (Fig. 2E), while PDE8A was present in CL and in follicles (Fig. 2F). Strong staining for PDE11A was observed in oocytes (Fig. 2G).

Follicle activation

No effect of the two PDE4 inhibitors LEO29102 and LEO28386 was seen on the distribution of follicle classes ($\chi^2=10.7$, $P=0.09$) after 1 week of culturing as compared to controls (Fig. 3). The mean follicle diameter was lower in the LEO29102 ($17.9\pm0.11\ \mu\text{m}$) and LEO28386 ($17.8\pm0.11\ \mu\text{m}$) groups as compared to control ($18.3\pm0.10\ \mu\text{m}$, $P<0.01$ for both groups). In addition, no effect was seen on the expression of *Kitlg*, *Inha*, the growing follicle markers *Gdf9*, *Amh*, the proliferation marker *Mki67* or the primordial follicle marker *Tmeff2* (Fig. 4). The cAMP analogue 8-bromo-cAMP increased *Mki67*, *Inha*, *Tmeff2* and decreased *Amh* in a dose-dependent manner, while *Gdf9* and *Kitlg* were unchanged after 1 week of treatment.

In the ovaries cultured for 6 h, no nuclear export of FOXO3A was observed following exposure to 1 mM 8-bromo-cAMP, or 1 μM of the PDE4 inhibitor LEO29102 or LEO28386 (Fig. 5). Furthermore, western blot analysis was unable to detect phosphorylation of FOXO3A or AKT1 in ovaries exposed to 100 μM 8-bromo-cAMP for 6 h (Fig. 6).

Discussion

This study showed that all cAMP hydrolysing PDEs families were expressed in the rat ovary with *Pde3a*, *Pde7a/b* and *Pde8a* displaying the highest expression. *Pde4d* was the major PDE type 4 subtype present in the rat ovary but accounted for a modest 0.5–3.2% of the total cAMP PDE mRNA. In contrast, the PDE4 enzymatic activity was between 15 and 26% of the total PDE activity in GC.

Figure 3 Follicle classification of cultured neonatal rat ovaries. The ovaries were cultured for 7 days *in vitro* in presence of the PDE4 inhibitor LEO29102 1 μM or LEO28386 1 μM , or control (DMSO). Error bars indicates S.E.M. for $n=3$.

Figure 4 Gene expression of growth and maturation markers in cultured neonatal rat ovaries. The ovaries were cultured for 7 days *in vitro* in presence of the PDE4 inhibitor LEO29102 1 μ M or LEO28386 1 μ M, or control (DMSO). In addition, the effect of various concentration of the cAMP analogue 8-bromo-cAMP was tested. The following genes were measured: inhibin A, alpha subunit (Inha), growth differentiation factor-9 (Gdf9), anti-Müllerian hormone (Amh), Kit ligand (Kitlg), antigen KI-67 (Mki67) and transmembrane protein with EGF-like and two follistatin-like domains 2 (Tmeff2). The gene expression was standardized to the house keeping gene Rpl32 and normalized to the control group. Error bars indicate S.E.M. * P <0.05; ** P <0.01; *** P <0.001 vs control.

The presence of a number of different PDE families in the ovary and in the follicles allow cells to regulate cAMP tightly at various concentrations due to differences in and post-translational modifications and affinity for cAMP. Both PDE7 and PDE8 are most active at low cAMP concentrations (K_m < 100 nM) and PDE8 has been suggested to regulate the resting levels of cAMP (Bender & Beavo 2006, Tsai *et al.* 2011, Shimizu-Albergine *et al.* 2012) and it can be speculated that PDE7 and PDE8 share the same function in the ovary. In contrast, PDE4 has a lower affinity for cAMP (K_m = 1–10 μ M) meaning that this enzyme is mainly active at higher cAMP

concentrations observed during hormonal stimulation of cAMP production as seen for instance with hCG stimulation (Park *et al.* 2003, Bender & Beavo 2006). This may explain why we were unable to observe a stimulatory effect on primordial follicle activation when inhibiting PDE4. Perhaps the cAMP concentration was too low for optimal PDE4 activity.

Both PDE3 and PDE2 hydrolyse cAMP but are both affected by cGMP although they differ in their effect since PDE2 is stimulated while PDE3 is inhibited by cGMP (Azevedo *et al.* 2014). The latter is important in the oocyte of the preovulatory follicle, where PDE3 activity

Figure 5 Immunolocalization of FOXO3A after treatment with 8-bromo-cAMP or PDE4 inhibitors. Neonatal ovaries were treated with 1 mM 8-bromo-cAMP (B), 1 μM LEO28386 (C), 1 μM LEO29102 (D), DMSO (A) for 6 h or no treatment (F) followed by staining for FOXO3A. Staining with no primary antibody was used as control (F). No nuclear export of FOXO3A was seen in any of the groups. Scale bar = 100 μm.

is inhibited by cGMP originating from the cumulus cells. After the LH peak, cGMP declines and the PDE3 activity increases leading to a reduction in the cAMP concentration in the oocyte and resumption of meiosis (Vaccari *et al.* 2009). Although the function of cGMP in the CL is unclear (Budnik *et al.* 1987, Johnson *et al.* 1999), the presence of PDE2 suggests that cGMP may diminish the effect of cAMP by increasing its degradation.

We saw a relative low expression of *Pde4d* compared to the enzymatic activity of PDE4. Recently, similar results have been reported in human GCs (Petersen *et al.* 2015). This could be due to a rapid turnover of the mRNA as seen with other transcripts involved in signalling (Schwanhauser *et al.* 2011). This would be advantageous as it allows a fast response to changes in cAMP concentration, e.g. as seen in conjunction with hCG stimulation (Park *et al.* 2003). Despite not accounting for the majority of the PDE activity in the GC, PDE4 is well known to exert important functions in the rodent ovary (Park *et al.* 2003, McKenna *et al.* 2005, Losco *et al.* 2010). This may be caused by the previous mentioned differences in cAMP affinity or the subcellular distribution of the PDE families (Conti *et al.* 2014).

We found high levels of *Pde3a* in the small follicles. This is in line with previous reported *in-situ* hybridization of rat ovaries where *Pde3a* and to a lesser degree *Pde3b* were located in the oocytes (Reinhardt *et al.* 1995). However, it is unclear what the function is in the preantral follicles as the cumulus cells that are essential

in controlling the PDE3 activity in the oocyte of larger follicles are not developed at this time.

Low level of *Pde8b* was seen in contrast to that observed in bovine ovaries (Sasseville *et al.* 2009) and in humans (Markholt *et al.* 2012, Petersen *et al.* 2015). Instead, *Pde8a* appeared to be the major subtype present, especially in the CL. A higher level of *Pde2a* was also seen in the CL supported by the IHC staining. The IHC staining suggested high levels of PDE11A in the oocyte although the mRNA expression was very low in the small preantral follicles.

We saw no effect of the tested PDE4 inhibitors on the *in vitro* follicle activation after 7 days of culture, neither in the distribution of the different follicle stage in the histological sections nor in genes differentially expressed in the folliculogenesis. This is in contrast to previous reports of such an effect of cAMP analogues in humans (Zhang *et al.* 2004). Neither the PDE4 inhibitors nor 8-bromo-cAMP affected the expression of *Kitlg*. This is in line with a previous report that cAMP does not stimulate KITLG in GC (Grimaldi *et al.* 2003) but in contrast to findings from ovarian cancer cells (Shaw *et al.* 2002). The lack of effect on *Inha* expression suggests that PDE4 inhibition alone is not able to generate a sufficient elevation of cAMP to cause a biological effect in the neonatal ovaries as the high dose 8-bromo-cAMP did increase *Inha* expression.

The tumour suppressor TMEFF2 has recently been shown to be highly expressed in oocytes isolated from human primordial follicles (Markholt *et al.* 2012). Although traditional markers of the preantral follicle stage like *Gdf9* and *Amh* were not augmented in response to 8-bromo-cAMP stimulation, *Tmeff2* expression was dose dependently increased as a result of exposure to 8-bromo-cAMP stimulation. The precise function of TMEFF2 in the early follicle is unknown, but it has been shown to be down-regulated in cancer cells and to inhibit platelet-derived growth factor (PDGF) AA, that stimulates follicle growth (Sleer & Taylor 2007, Lin *et al.* 2011, Brito *et al.* 2012, Chen & Ruiz-Echevarria 2013). This suggests that TMEFF2 may play a role in maintaining the primordial follicle in a quiescent state.

Figure 6 Protein expression of phospho-AKT1 S473, phospho-FOXO3A S253 and FOXO3A after treatment with 8-bromo-cAMP. Western blot of neonatal ovaries cultured for 2 h in presence of 100 μM 8-bromo-cAMP. Rat tongue was used as positive control for phospho-AKT1. 40 μg total protein was used for rat tongue and PN8 ovaries and 13 μg total protein for PN6 ovaries.

In corticotroph cells, TMEFF2 inhibits the cAMP production from CRH (Labeur *et al.* 2010), suggesting that the increased expression of *Tmeff2* may act as a negative feedback on the increase cAMP level.

We cannot exclude that additional growth factors stimulating cAMP production other than the 25 U/l FSH and 1% FBS may be required in the culture media to obtain a sufficient high cAMP level for PDE4 inhibitors to be effective. Alternatively, inhibition of additional PDEs may be required. Based on the distribution in the small follicles PDE3, PDE7 and PDE8 seem to be obvious candidates.

In the conditions applied in this study, 8-bromo-cAMP did not increase phosphorylation of AKT1, an early marker of follicle activation. The effect of cAMP on the PI3Kinase pathway has been described to differ between cell types as the two main effectors of cAMP: protein kinase A and EPAC have been reported to have opposing effects on the pathway (Smith *et al.* 2005, Chen *et al.* 2007, Kwak *et al.* 2008, Baviera *et al.* 2010). In the present set of experiments, the effect of cAMP seems to be inhibitory on the FOXO3A/AKT1 phosphorylation. An explanation for the previous reported effects of cAMP on primordial follicle activation (Zhang *et al.* 2004) could be due to changes in hormone/growth factor secretion from later follicular stages affecting the primordial follicles, as the proportion of secondary follicles was higher in the human study. Another important difference between the two studies is the inclusion of FSH in the culture media in the present study, as additional cAMP may not stimulate the follicle growth further (Ding *et al.* 2012). However, 8-bromo-cAMP did increase the expression of *Inha* suggesting that the ovaries were cAMP responsive. Finally, the culture circumstances in this study might not be optimal for follicular growth although media with similar composition as ours has been used successfully for culturing of rodent ovaries (Wang *et al.* 2013).

It is well known, that there are species differences in the expression of PDEs in various organs (Bian *et al.* 2004, Johnson *et al.* 2012). This poses a problem when translating results obtained in one species to another. The data obtained in this study are likely to prove useful in interpreting data obtained in rat ovaries as information of all PDEs in the ovary has been lacking until now. In addition, the high expression of PDE7 and PDE8 suggests that inhibitors of these families could have an effect on the female reproductive system in rats.

In conclusion, this study showed that in addition to the already known PDE3 and PDE4, the two low K_m PDEs, PDE7 and PDE8 are present in rat ovaries in significant amounts. We did not find any effect of PDE4 inhibition on primordial follicle activation in cultured *in vitro* neonatal rat ovaries. In addition, 8-bromo-cAMP failed to activate the PI3Kinase/AKT pathway, suggesting that cAMP in itself has no direct effect on initiation of primordial follicles growth.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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