

# FSH-induced phosphoprotein phosphatase 2A-mediated deactivation of particulate phosphodiesterase-4 activities is abolished after alteration in proteoglycan synthesis in immature rat Sertoli cells

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## Abstract

Cessation of rat testicular Sertoli cells proliferation around days 15–20 *post partum* is associated *in vitro* with the highest rise in rolipram-sensitive cAMP-catabolizing phosphodiesterase-4 activities (PDE4s) triggered by FSH during the early postnatal period. The transient nature of FSH-induced increase in PDE4s suggests concomitant changes in both PKA-mediated activation and subsequent deactivation of these activities. In this study, we demonstrated that the deactivation of FSH-stimulated particulate, but not soluble, PDE4s in cultured Sertoli cells from 20-day-old rats was inhibited by phosphoprotein phosphatase (PP) inhibitors, okadaic acid, and calyculin A. Moreover, the deactivation of FSH-stimulated particulate PDE4s was timely related with the gonadotropin-induced increase in both particulate PP2A activity and particulate PP2A catalytic subunit immunoreactive expression independently of any transcriptional regulation of that subunit. Both the FSH-induced increase in

recruitment/activation of particulate PP2A and the subsequent deactivation of particulate PDE4 were abolished when Sertoli cell proteoglycans (PGs) synthesis was altered by sodium chlorate. Sodium chlorate effect was developmentally regulated as evidenced by its ability to silence particulate PDE4 deactivation only in non-proliferating (from 20- to 30-day-old rats) but not in proliferating (from 10-day-old rats) Sertoli cells. All these data suggested that PGs could be involved in the FSH-induced recruitment/activation of PP2A. Particularly, developmentally regulated transmembrane syndecans, the most abundant PGs in Sertoli cells, by targeting PP2A at the membrane level could allow developmental control of activated particulate PDE4s and, potentially, other signaling phosphoproteins, including the FSH receptor, during the early postnatal period.

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## Introduction

Phosphodiesterases (PDEs) that catalyze hydrolysis of cAMP and cGMP are potent regulators of intracellular concentrations of these second messengers. Among the 11 subfamilies (from PDE1 to PDE11) that differ in terms of substrate, inhibitor specificities, and molecular sequence (Bender & Beavo 2006, Lugnier 2006, Conti & Beavo 2007), the cAMP-specific PDE4 subfamily is of primary importance since its members are subject to cAMP feedback regulation mediated through protein kinase A (PKA)-mediated phosphorylation and transcription (Houslay & Adams 2003). PDE4s are encoded by four genes generating more than 25 spliced variants that have been divided into long, short, and super-short isoforms according to the presence or absence of two conserved N-terminal sequences called upstream conserved region (UCR)-1 and UCR-2, which are thought to play a determinant role in their intracellular targeting (Baillie *et al.* 2005). In testicular Sertoli cells, PDE4D have been recognized as the main cAMP-catabolizing enzymes (Morena *et al.* 1995) regulating the response to the gonadotropin

follicle-stimulating hormone (FSH) that controls the acquisition of differentiated functions. Although FSH triggers multiple signals in Sertoli cells (Walker & Cheng 2005), the cAMP pathway constitutes the primary route for FSH action. Increase in cAMP intracellular concentrations is tightly controlled by simultaneous increase in PDE4 activities resulting from PKA-mediated serine phosphorylation of long isoforms and cAMP-mediated transcriptional up-regulation of short isoforms (Richter *et al.* 2005). In rat Sertoli cells, transition from proliferative to quiescent and differentiated phenotype during the early postnatal period (between 15 and 20 days *post partum*) is associated with switching of FSH receptor from mitogen activated protein kinase (MAPK)3/1 activation to adenylate cyclase activation (Crepieux *et al.* 2001). The turning age of 20 days *post partum* is also characterized by both the highest cAMP response to FSH and the highest particulate and soluble PDE4s activation by the gonadotropin without changes in subcellular distribution and expression of PDE4D proteins (Levallet *et al.* 2007b). Thus, the amplification of transient response in both subcellular fractions at day 20 in Sertoli cells

suggests developmental changes not only in PKA-mediated phosphorylation of long PDE4D isoforms but also in their subsequent deactivation by a putative phosphatase activity.

Very little is known about serine/threonine protein phosphatases involved in the regulation of phosphorylation of PDE4D long isoforms. To date, only deactivation of PDE3B has been shown to involve phosphoprotein phosphatase 2A (PP2A) in rat adipocytes (Resjo *et al.* 1999). PP2A, a dual serine/threonine and tyrosine phosphatase, through its ability to dephosphorylate number of signaling proteins, is thought to be associated with growth inhibition and cell arrest (Janssens *et al.* 2005). Most of the cellular PP2A holoenzymes are in trimeric form, in which a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit are associated with a particular regulatory B subunit (Janssens & Goris 2001). The nature of PP2A-B subunit determine both substrate specificity and subcellular localization of the holoenzyme (Janssens & Goris 2001, Strack *et al.* 2004, Janssens *et al.* 2005) through the formation of signaling complexes in which both protein kinase and phosphatase are often associated (Millward *et al.* 1999, Wong & Scott 2004). Cell proteoglycans (PGs) appear as potential candidates to anchor such signaling enzymes as suggested by the alteration of FSH-induced particulate PDE4 activities triggered by the inhibition of PG synthesis in rat Sertoli cells (Levallet *et al.* 2007a).

PGs are complex molecules composed of sulfated glycosaminoglycans covalently linked to a protein core and are present either in extracellular matrix or associated with cells (Prydz & Dalen 2000). Cell heparan sulfate PGs (HSPGs) have been involved in numerous processes, such as cell adhesion, cell growth, and cell survival (Bernfield *et al.* 1999, Tumova *et al.* 2000). Syndecans are the major HSPGs expressed at the cell surface and the four members of the family (designated syndecans 1–4) identified in vertebrates (Carey 1997) display highly conserved transmembrane and cytoplasmic domains (Tkachenko *et al.* 2005). Albeit devoid of intrinsic catalytic activity, the presence of an identical COOH-terminal postsynaptic density 95, disk large, zona occludens-1 (PDZ)-binding motif known to bind at least four PDZ domain-containing proteins (Tkachenko *et al.* 2005) suggests that syndecans may recruit some signaling complexes at the membrane level.

Use of specific chemical inhibitors of PG synthesis such as sodium chlorate, an inhibitor of the active sulfate donor for sulfotransferases (Baeuerle & Huttner 1986), and para-nitrophenyl- $\beta$ -D-xyloside (PNPX), a competitive inhibitor of endogenous core protein for the elongation of glycosaminoglycan chains (Carey 1991), offers a valuable tool to explore the role of cell PGs in signaling functions. In Sertoli cell cultures, both inhibitors reduce drastically the amount of cell PGs and simultaneous addition of FSH and PNPX or sodium chlorate induces increase in the FSH-stimulated estradiol synthesis (Phamantu *et al.* 1995, 1999) consequently to a decrease in particulate PDE4 activities (Levallet *et al.* 2007a). However, due to the half-life (about 12 h) of Sertoli cell PGs (Fagnen *et al.* 1999) which are mainly constituted by transmembrane HSPGs (Brucato *et al.* 2001), it is likely that

early response of Sertoli cell to FSH is probably unaffected by chlorate or PNPX treatment in these experiments. Thus, we have re-evaluated the incidence of alteration of PG synthesis by pre-treating Sertoli cell with sodium chlorate for 24 h before FSH stimulation. This study describes the alteration of deactivation of FSH-stimulated particulate PDE4 activities under chlorate conditions and identified the abolition of FSH-stimulated particulate PP2A-like PP as the main effect induced by the decrease in cell PG content.

## Materials and Methods

### Materials

Ovine FSH (oFSH-20, 4453 IU/mg) was kindly provided by the National Institute of Arthritis, Metabolic and Digestive Diseases (Pituitary Hormone Distribution Program, Bethesda, MD, USA). Ultrosor SF (steroid-free serum substitute) was purchased from BioSeptra (Cergy-Saint-Christophe, France). Okadaic acid (OA) and calyculin A (CA) were from Calbiochem (Nottingham, UK). [2,8-<sup>3</sup>H] Adenosine 3',5'-cyclic phosphate (1.59 TBq/mmol) was from Amersham Biotech. Cyclic AMP (<sup>3</sup>H) assay system and enhanced chemiluminescence (ECL) staining kit were from GE Healthcare Europe (Orsay, France). PDE4D (K-16) and PP2A (C-20) antibodies, blocking peptide, and horse radish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), monoclonal mouse anti-actin (Ab-1) from Calbiochem (Darmstadt, Germany). All others reagents were of analytical grade.

### Cell cultures

All procedures described within were performed in accordance with French legal requirements for animal handling and welfare. After the killing of 10- to 30-day-old Sprague-Dawley rats (provided by Elevage Janvier, Le Genest Saint-Isle, France), Sertoli cells were isolated from decapsulated testes by sequential enzymatic digestion at 32 °C (trypsin, then collagenase/dispase plus hyaluronidase) as previously described (Levallet *et al.* 2007b) and cultured at 32 °C under 5% CO<sub>2</sub> atmosphere (v/v) for 48 h in Ham's F12–Dulbecco's modified Eagle's medium (1:1) supplemented with antibiotics (50 000 UI/1 penicillin, 50 mg/1 streptomycin and 50 mg/1 kanamycin), fungicide (0.25 mg/1 amphotericin B), 2.2 g/1 sodium bicarbonate, and 2% Ultrosor SF (v/v). Seeding Sertoli cell densities and contamination by peritubular and germ cells have been previously described (Levallet *et al.* 2007b).

On day 3 after plating, residual germ cells were lysed by hypotonic treatment using 20 mM Tris–HCl buffer (pH 7.4; Galdieri & Zani 1981). Rat Sertoli cells (10- to 30-day-old) were then cultured for additional period (up to 2 days) in medium devoid of Ultrosor. Sertoli cell cultures were routinely assayed for peritubular cell contamination by checking the lack

of histochemical detection of pericellular alkaline phosphatase and absence of fibronectin expression by RT-PCR as previously described (Levallet *et al.* 2007a,b). On day 4 after plating, 10- to 30-day-old rat Sertoli cells were incubated for 24 h in the presence or absence of 10 mM sodium chlorate. At the end of the incubation, the cell medium was renewed and Sertoli cells were further incubated with 100 ng/ml FSH for up to 24 h and in the presence or absence of CA or OA before being assayed for cAMP-PDE and PP2A activities, total RNA extraction, or immunoblotting experiments.

#### Extraction of total RNA and RT-PCR analysis

Total RNA was isolated from Sertoli cell cultures (20-day-old rats) with an acid guanidinium thiocyanate-phenol-chloroform (Chomczynski & Sacchi 1987). RNA were reverse-transcribed into cDNA using Moloney-murine-leukaemia-virus reverse transcriptase and used as templates for PCRs (Levallet *et al.* 2007a,b). The primers included 5'-TACTCC-GAGGGAATCACGAG-3' and 5'-CTCGGATGTGATC-CAGTGTG-3' for *Pp2a-Cα* (Accession no. NM\_017039), 5'-GGCCCAATGTGTGATCTCTT-3' and 5'-CCATTA-CAAGCTGGTGAGCA-3' for *Pp2a-Cβ* (NM\_017040), 5'-ACAGACTACCTCATGAAGAT-3' and 5'-AGCCAT-GCCAAATGTCTCAT-3' for β-actin (V01217). According to the PCR primer set, the following conditions were used: denaturation at 94 °C for 1 min, annealing at 55 °C (β-actin) or 60 °C (*Pp2a-C*) for 45 s, and extension at 72 °C for 1 min for 35 and 22 cycles for PP2A and Actin amplification respectively. PCR products were separated by electrophoresis in a 2.5% agarose gel (w/v) and stained with ethidium bromide. Gels were photographed under u.v. illumination, and densities of the bands were measured using NIH Image J software (National Institute of Health, Bethesda, MD, USA).

#### Preparation of cell fractions

Cultured Sertoli cells from 10-, 20-, and 30-day-old rats were washed with cold PBS and recovered by gentle scraping in lysis buffer (20 mM Tris-HCl pH 7.2, 1 mM EDTA, 250 mM sucrose) supplemented with 0.1 mM phenylmethanesulfonyl fluoride, 2 mM benzamide, and a mixture of antiproteases (antipain, aprotinin, leupeptin, pepstatin A) at a final concentration of 1 µg/ml and centrifuged at 100 000 g for 1 h at 4 °C, as previously described (Levallet *et al.* 2007a,b). The supernatant (soluble fraction) and the pellet (particulate fraction) were then stored at -20 °C before the determination of total and rolipram-sensitive PDE activities. The purity of each subcellular fraction was checked by assaying both lactate dehydrogenase (soluble activity) and alkaline phosphatase (membrane-associated activities) as has been previously described (Levallet *et al.* 2007b).

#### PDE assay

PDE activities were assayed according to the two-step modified procedure of Thompson & Appleman (1971).

Protein mixture (10 µg) was incubated at 34 °C for 15 min in 200 µl of reaction buffer (40 mM Tris-HCl (pH 8.1), 1 mM MgCl<sub>2</sub>, 1.25 mM β-mercapto-ethanol, 0.14 mg BSA) in the presence of 1 µM camp ([2,8-<sup>3</sup>H]-cAMP (3.7 kBq/tube)). To identify the cAMP-PDE4 activity, a specific inhibitor, rolipram, was added to the incubation mixture at a final concentration of 10 µM. The PDE activity measured in the presence of rolipram was subtracted from the total activity to quantify the PDE4 activity.

The reaction was then stopped by the addition of 200 µl of stop solution (40 mM Tris-HCl (pH 7.5), 10 mM EDTA) followed by heat denaturation at 100 °C for 1 min. *Crotalus atrox* snake venom (50 µg) was added to each sample and incubated at 34 °C for 20 min. The reaction products were separated by anion exchange chromatography using a freshly prepared solution of Dowex (Dowex/water/ethanol: 1/1/1: w/v/v) and an aliquot of supernatant was then counted by liquid scintillation (Levallet *et al.* 2007a,b).

#### PP2A activities

Total PP2 activities in subcellular fractions of cultured Sertoli cells from 20-day-old rats were determined by serine/threonine phosphatase assay system (Promega) containing a synthesized phosphopeptide, RRA(pT)VA compatible with serine/threonine phosphatases 2A, 2B, and 2C but poor substrate for protein phosphatase 1. Assays were conducted according to the manufacturer's procedure using suitable PPase reaction buffer (250 mM imidazole (pH 7.2), 0.1% β-mercaptoethanol (v/v), 1 mM EGTA, and 0.5 mg/ml BSA) with or without PP2A inhibitor okadaic acid (50 nM). Substrate-released phosphate was quantified by colorimetric method, and PPase activities were expressed first as pmol/min per mg of incubated protein then as nmol/min per subcellular fractions.

#### Western blotting

Proteins (25 µg) of each subcellular fraction obtained from 20-day-old Sertoli cells were boiled for 5 min and separated by SDS (8%: (w/v))/PAGE. Proteins were transferred onto nitrocellulose membrane 1 h at 100 V, 4 °C. Western blots were then performed using affinity-purified goat polyclonal antibodies PDE4D (K-16), raised against a peptide mapping near the C-terminus of PDE4D of human origin, and PP2A (C-20) antibody raised against a recombinant protein corresponding to amino acids 1-309 representing full-length protein PP2A catalytic subunit (PP2Ac) of human origin, or monoclonal mouse anti-actin (Ab-1). Immunoreactive bands were detected using the donkey anti-goat IgG-HRP (for PDE4D and PP2A primary antibodies) or goat Anti-mouse IgM, H&L chain-specific peroxidase conjugate and ECL staining kit. The western blot was scanned on ProXPRESS Proteomic Imaging System (Perkin-Elmer Life Science, Boston, MA, USA) and analyzed with TotalLab Image Analysis software (Nonlinear Dynamics Ltd, Newcastle, UK).

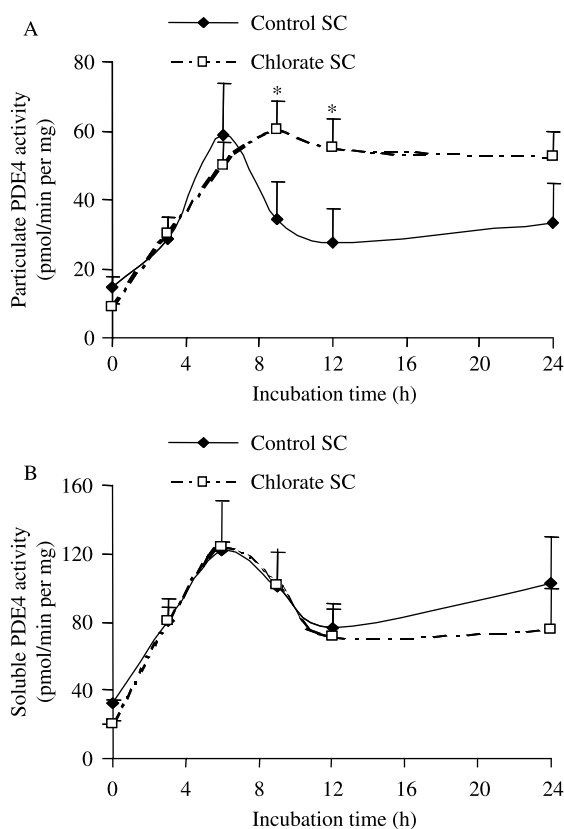
### Statistical analysis

Differences between treatments were determined by one-way ANOVA followed by Fisher's test using the Sigma Stat software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at  $P < 0.05$ .

## Results

### Alteration of cell PG by sodium chlorate prolonged the FSH-induced stimulation of particulate PDE4 activities in 20-day-old Sertoli cells

Addition of FSH to Sertoli cell cultures stimulated rolipram-sensitive PDE4 activities in both particulate and soluble fractions reaching peak levels (approximately fourfold the initial values) after 6 h incubation (Fig. 1A). Further duration of incubation led to a progressive decrease in these activities without achieving initial levels particularly for soluble PDE4 activities. In contrast, no stimulation of rolipram-insensitive cAMP-PDE activities

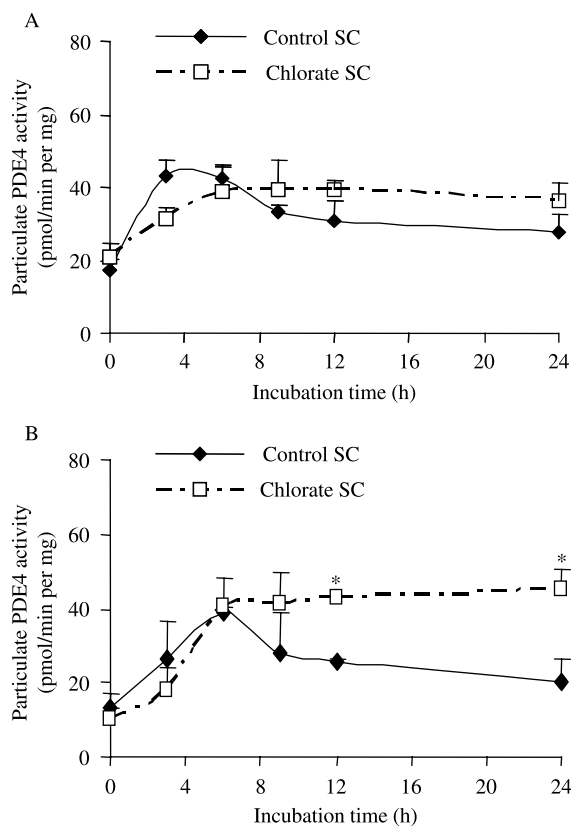


**Figure 1** Kinetics of FSH-stimulated particulate (A) and soluble (B) PDE4 activities in untreated and chlorate-treated Sertoli cells (SC) from 20-day-old rats. Sertoli cells were pre-treated (chlorate SC) or not (control SC) with 10 mM sodium chlorate for 24 h before challenge with FSH (100 ng/ml) for up to 24 h. PDE4 activities were expressed in pmol cAMP hydrolyzed per min per mg protein. Values are means  $\pm$  S.E.M. of three independent experiments. \*Significantly different ( $P < 0.05$ ) from control SC.

( $84.5 \pm 2.3$  and  $71.0 \pm 3.5\%$  of total PDE activities in soluble and particulate fractions respectively) occurred in both subcellular compartments in the presence of gonadotropin (data not shown).

Pre-incubation of cells with sodium chlorate did not alter significantly basal and FSH levels of both particulate and soluble PDE4 activities at least during 6 h after the onset of the stimulation. However, in chlorate-treated Sertoli cells, the decrease in FSH-stimulated particulate PDE4 activities recorded after 6 h was totally suppressed (Fig. 1A) whereas the pattern of FSH-stimulated soluble PDE4 activities was unaffected (Fig. 1B) as well as those of rolipram-insensitive PDE activities in both subcellular compartments (data not shown).

A similar effect, although of lesser amplitude, was observed when the alteration of cell PG was induced by pre-incubation for 24 h in the presence of 1 mM PNPX, a competitive inhibitor of endogenous core protein for the elongation of glycosaminoglycan chains (data not shown). Moreover, pre-treatment of Sertoli cells with 10 mM NaCl or 10 mM PNPX, and the structural analogues of sodium chlorate and



**Figure 2** The ability of sodium chlorate to alter time course of FSH-stimulated particulate PDE4 activities in Sertoli cells (SC) is developmentally regulated. Sertoli cells from (A) 10- and (B) 30-day-old rats were pre-treated (chlorate SC, dashed lines) or not (control SC, solid lines) with 10 mM sodium chlorate for 24 h, then further stimulated with FSH (100 ng/ml) for up to 24 h. PDE4 activities were expressed in pmol cAMP hydrolyzed per min per mg protein. Values are means  $\pm$  S.E.M. of three (10 days) and four (30 days) independent experiments. \*Significantly different ( $P < 0.05$ ) from control SC.

PNPX respectively, which were ineffective upon cell PG synthesis (Phamantu *et al.* 1995, 1999), did not modify the kinetic pattern of FSH-stimulated PDE4 activities (data not shown). All these data suggest that sodium chlorate effect was specific and related to its ability to decrease cell PG content.

*The ability of sodium chlorate to prolong FSH-induced stimulation of particulate PDE4 in immature rat Sertoli cells was developmentally regulated*

To strengthen the hypothesis that alteration of cell PGs were involved in the sustained activation of FSH-stimulated particulate PDE4, we have performed similar experiments with Sertoli cells originating from 10- and 30-day-old rats. Differentiation of Sertoli cells was associated to the loss of their ability to divide around days 15–20 *post partum* (Clermont & Perey 1957), a process often associated with the HSPG enrichment of cell membrane in many cell types (Leppa *et al.* 1992, Kato *et al.* 1995, Inki & Jalkanen 1996). Although FSH stimulated particulate PDE4 activities in both proliferating (10 days) and quiescent Sertoli cells (30 days; Fig. 2), sodium chlorate had the ability to prolong FSH stimulation only in 30-day-old Sertoli cells. In both cell types, sodium chlorate did not affect the kinetic pattern of FSH-stimulated PDE4 activities in soluble fraction (data not shown). This set of experiments showed that the ability of sodium chlorate to alter the pattern of PDE4 activation under FSH conditions was developmentally regulated and could be related to higher expression of HSPGs in quiescent Sertoli cells.

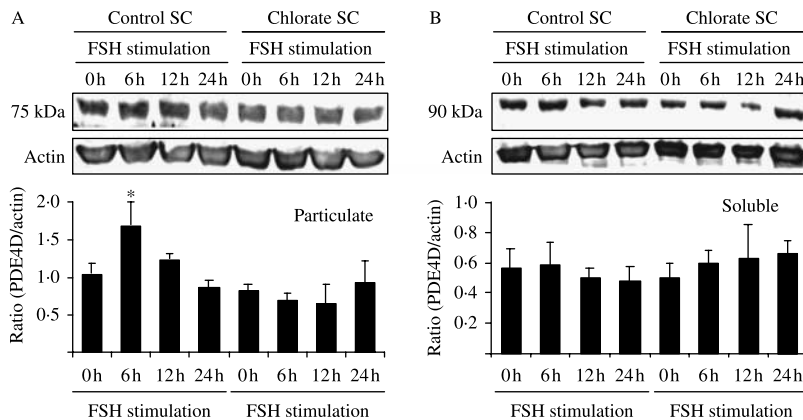
*Long PDE4D isoforms expression was unaffected by sodium chlorate treatment in 20-day-old rat Sertoli cells*

To investigate whether the distribution and/or protein levels of PDE4D isoforms were altered in sodium chlorate-treated cells,

subcellular fractions from untreated and chlorate-treated Sertoli cells challenged with FSH were immunoblotted with anti-human PDE4D antibody which has been demonstrated as suitable for detection of rat PDE4Ds. Our previous results have shown that short PDE4D1-like immunoreactive 75 kDa protein was the main PDE4D present in particulate fraction whereas 90 kDa long PDE4Ds, corresponding to PDE4D3 and PDE4D9 isoforms expressed in Sertoli cells, predominated in soluble fraction (Levallet *et al.* 2007a). As illustrated in Fig. 3, chlorate pre-treatment of Sertoli cells did not alter the expression pattern of immunoreactive 90 kDa proteins in both subcellular fractions but suppressed the FSH-induced up-regulation of PDE4D1 immunoreactive protein in particulate fraction. The other immunoreactive bands (68, 100, and 110 kDa) present in particulate fraction (Levallet *et al.* 2007a) did not display significant changes in the same conditions (data not shown). Thus, changes in protein levels of PDE4Ds could not account for the sustained PDE4 activities in chlorate-treated Sertoli cells. All these data suggested that the bell-shaped curve of particulate PDE4 activities under FSH conditions mainly resulted from a series of post-translational modifications of long PDE4D isoforms and that chlorate pre-treatment altered exclusively the later step(s) leading to a decrease in particulate PDE4 activities.

*PP2A inhibitors mimicked the sustained stimulation of FSH-induced particulate PDE4 activities recorded in sodium chlorate-treated cells*

Since the activation of long PDE4 isoforms was known to result from PKA-induced phosphorylation of Ser residue (Richter *et al.* 2005), we have hypothesized that decrease in PDE4 activities could be the result of dephosphorylation process induced by a serine/threonine phosphatase. To determine the role of phosphatase activities and their nature,



**Figure 3** Effects of chlorate pre-treatment on particulate 75 kDa short PDE4D1 and soluble long 90 kDa PDE4Ds protein expressions in Sertoli cells (SC) challenged with FSH. (A) Particulate and (B) soluble fractions were prepared from 20-day-old rat cultured Sertoli cells first pre-treated for 24 h with (chlorate SC) or without (control SC) 10 mM sodium chlorate, then stimulated with 100 ng/ml of FSH for the indicated period. Western blot analyses were performed with polyclonal PDE4D antibody against a C-terminal region common to all PDE4D isoforms. Ratios of PDE4D expression to  $\beta$ -actin loading standard were determined in three separate cultures. Values are mean  $\pm$  s.e.m. \*Different from 0 h values at  $P < 0.05$ .

20-day-old rat Sertoli cells were incubated in the presence of FSH and 30 min before the peak of PDE4 activities (i.e. 5 h 30 min after the onset of experiment), phosphatase inhibitors CA and OA were added to the culture medium.

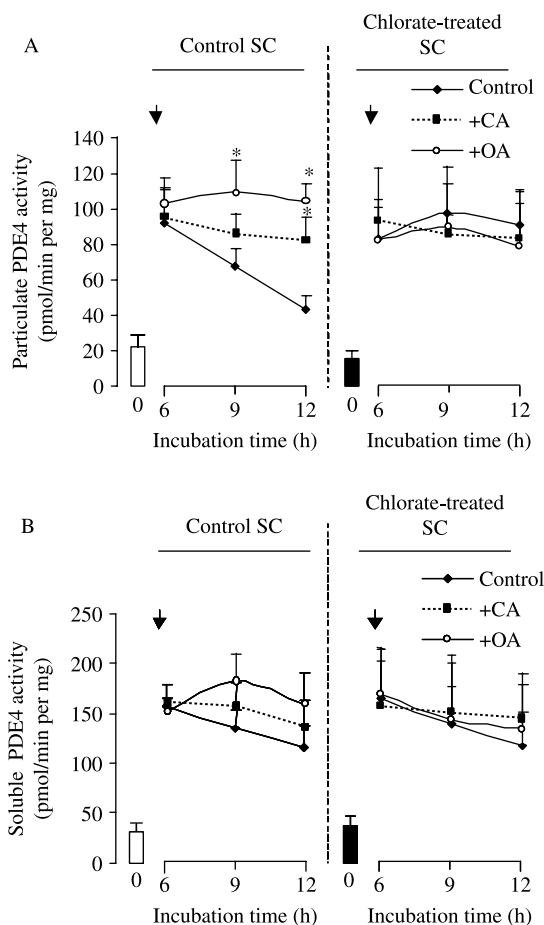
In untreated Sertoli cells, both CA and OA used at 1 nM prevented the decrease in FSH-stimulated particulate PDE4 activities observed after 6 h incubation, and sustained these activities at their maximal levels at least for 12 h (Fig. 4A). Higher concentrations (10 and 100 nM) of inhibitors gave the same results (data not shown) excepted for 100 nM CA which induced rapid rounding and cell detachment from the substratum, a phenomenon already observed for other cell types (Hosoya *et al.* 1993). In contrast, the deactivation of FSH-stimulated soluble PDE4 activities was not significantly affected by CA and OA (Fig. 4B), suggesting that mechanisms

or phosphatases responsible for the deactivation of PDE4 in both subcellular compartments were different.

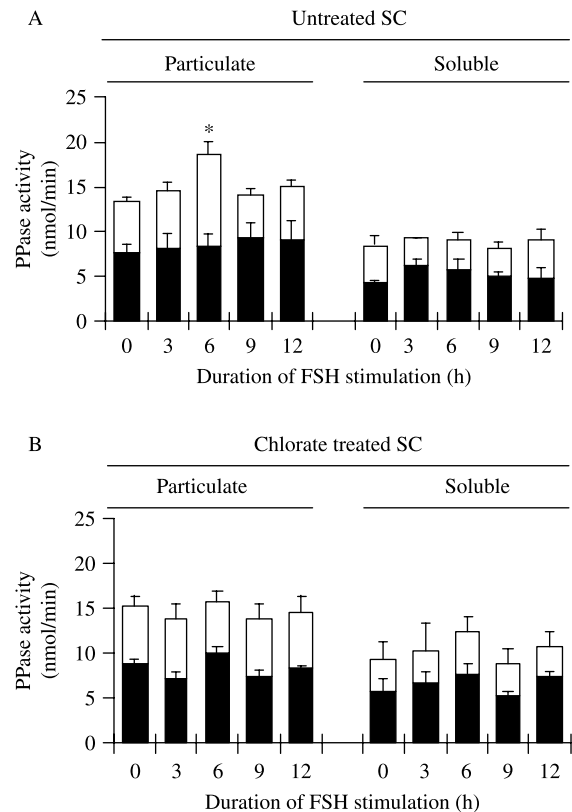
When Sertoli cells were pre-treated for 24 h with sodium chlorate, the sustained activation was not further modified by phosphatase inhibitors suggesting that sodium chlorate interfered with particulate PDE4 deactivation by inhibiting serine/threonine phosphatase identified as PP2A on the basis of similar potency of OA and CA to silence particulate PDE4 deactivation.

#### FSH-induced increase in particulate PP2A activity was abolished in sodium chlorate-treated Sertoli cells

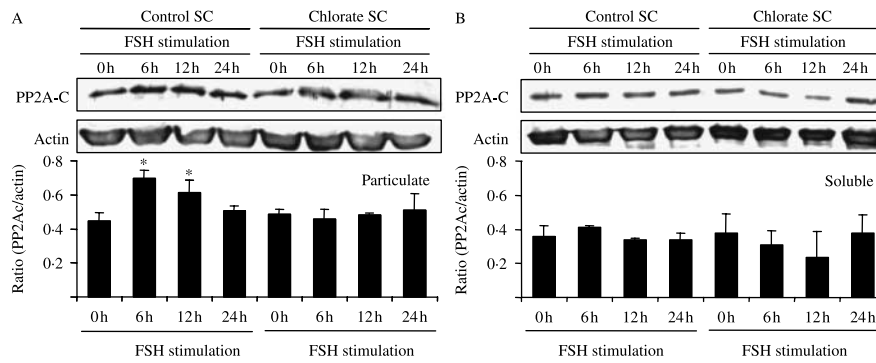
To further investigate the role of PP2A in the deactivation of particulate PDE4 activities, 20-day-old Sertoli cells were stimulated for specific times by the addition of FSH and analyzed for the activity and the distribution of PP2A in soluble and particulate fractions. According to the supplier,



**Figure 4** Effects of serine/threonine phosphoprotein phosphatase inhibitors upon kinetics of FSH-stimulated (A) particulate and (B) soluble cAMP-PDE4 activities in untreated and chlorate-treated Sertoli cell (SC) cultures from 20-day-old rat. Arrows indicated the time of addition of 1 nM okadaic acid (open circles) or 1 nM calyculin A (black squares). Values are means  $\pm$  S.E.M. of three independent experiments. \*Denotes significant effect of phosphatase inhibitor ( $P < 0.05$ ).



**Figure 5** The FSH-induced transient up-regulation of particulate PP2A activities is abolished in chlorate-treated Sertoli cells. Subcellular fractions of 20-day-old Sertoli cell cultures were then assayed for total PP2 activities using serine/threonine phosphatase assay system. Okadaic acid (50 nM)-sensitive PP2A activities (open bars) and other PP2 activities (black bars) were expressed in nmol of phosphate released per min. Values are means  $\pm$  S.E.M. of three independent experiments. \*Significantly different ( $P < 0.05$ ) from unstimulated Sertoli cells.



**Figure 6** The FSH-induced transient increase in particulate PP2A catalytic subunit expression is abolished in chlorate-treated Sertoli cells. Proteins samples from (A) particulate and (B) soluble fractions were obtained from 20-day-old Sertoli cells (SC) pre-treated (chlorate SC) or not (control SC) with 10 mM sodium chlorate and further stimulated for up to 24 h with 100 ng/ml FSH. Western blot analyses were performed with polyclonal anti-PP2A-C antibody densitometric quantifications were performed relative to  $\beta$ -actin signal. Values are means  $\pm$  S.E.M. of three experiments. \*Significantly different from control values at  $P < 0.05$ .

substrate used in PP2A assay was a poor substrate for PP1 (Donella Deana *et al.* 1990). Thus, the phosphatase activities determined with this assay were essentially PP2 activities and OA (50 nM)-sensitive PP2 activities were considered as PP2A.

In unstimulated Sertoli cells, nearly half of the total PP2 activities was supported by OA-sensitive PP2A activities. Addition of FSH to Sertoli cell cultures induced within 6 h a 60% increase in total particulate PP activity which was exclusively supported by OA-sensitive PP2A activity (Fig. 5). The increase in the particulate PP2A activity was transient and the return to initial levels was achieved 3 h later. The effect of FSH was restricted to the particulate fraction since no modification of soluble PP2A activity was recorded in the soluble fraction. Pre-treatment of Sertoli cells for 24 h with 10 mM sodium chlorate did not alter endogenous levels or subcellular distribution of PP2 activities but abolished the FSH-induced increase in particulate PP2A activities without apparent changes in soluble activities (Fig. 5).

#### *FSH-induced membrane recruitment of PP2A-C was abrogated in chlorate-treated Sertoli cells*

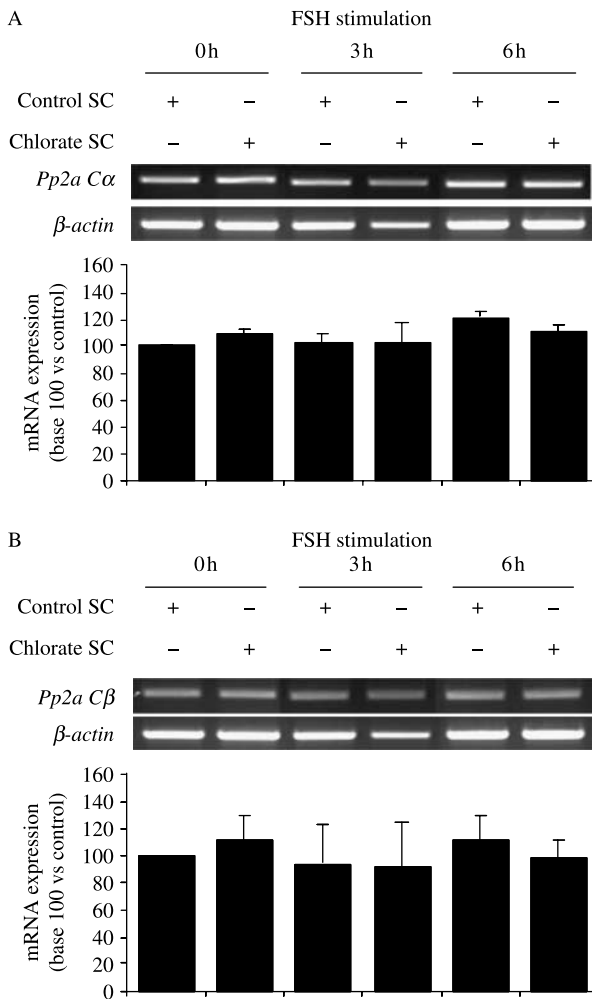
To investigate whether FSH promoted PP2A recruitment in the particulate fraction and whether chlorate pre-treatment altered such a recruitment, we performed a time course immunoblot analysis for the catalytic subunit of PP2A (PP2A-C). As shown in Fig. 6, the 36 kDa immunoreactive PP2A-C was detectable in both particulate and soluble fractions of 20-day-old Sertoli cells. No significant change has been recorded for PP2A-C levels in the soluble fraction of untreated Sertoli cells. However, particulate levels of PP2A-C increased (+30%,  $P < 0.05$ ) 6 h after FSH treatment, then slowly decreased to return to basal levels at 24 h. The FSH-induced increase in particulate PP2A-C immunoreactivity was totally abolished in chlorate-treated Sertoli cells while the expression of PP2A in soluble fraction was unaffected by chlorate pre-treatment.

#### *FSH-induced increase in particulate PP2A did not result from up-regulation of Pp2a-C expression*

To assess the involvement of up-regulation of *Pp2a-C* gene expressions in the FSH-induced increase in particulate PP2A protein, we sought a possible modification of steady-state mRNA for *Pp2a-C*. Since mammalian catalytic subunit of PP2A has two isoforms,  $\alpha$  and  $\beta$ , which are 97% identical and highly conserved (Mayer *et al.* 1990), we have evaluated the expression of both variants in Sertoli cells. Both isoforms were expressed in cultured Sertoli cells (Fig. 7). Chlorate pre-treatment has no effect on endogenous basal levels of mRNA for both isoforms, and FSH was unable to up-regulate their expression as well in untreated than in chlorate-treated Sertoli cells. Therefore, transcriptional regulation of catalytic subunit did not account for the changes in particulate PP2A immunoreactive expression and activity observed in our experiments.

## Discussion

This report shows that transient and inducible PP2A activity is responsible for the deactivation of FSH-stimulated particulate PDE4 activities in Sertoli cells and that effect is abolished after an alteration of cell PG synthesis. Thus, PKA-mediated phosphorylation of long PDE4 isoforms (Richter *et al.* 2005) and their PP2A-mediated dephosphorylation appear as the main mechanisms controlling particulate PDE4 activities. The abolition of FSH-induced up-regulation of particulate PDE4D1 immunoreactive expression (Fig. 3A) without concomitant changes in the peak values of PDE4 activities strongly suggests that PDE4D1 plays a minor role in the 6-h peak in cAMP-catabolizing activities triggered by FSH. However, that abolition may explain the accelerated return of PDE4D activities to baseline values recorded in our previous experiments when Sertoli cells were treated simultaneously with FSH and sodium chlorate (Levallet *et al.* 2007a).



**Figure 7** Lack of transcriptional regulation of (A) *Pp2a-Cα* and (B) *Pp2a-Cβ* expression by FSH in untreated and chlorate-treated 20-day-old rat Sertoli cells. Expression was semi-quantified by RT-PCR using specific primers. Densitometric data for each gene expression was normalized to the *β-actin* levels before expressed relative to endogenous level in untreated Sertoli cells. Means  $\pm$  S.E.M. from three different cultures are illustrated.

The precise mechanisms by which FSH itself limits excessive cAMP catabolism by reversing phosphorylation of particulate PDE4 activities are still unknown. Previous studies have shown that cAMP transiently up- or down-regulates PP2A activity in various cell types (Feschenko *et al.* 2002, Moon & Lerner 2003, Pullar *et al.* 2006), but the regulatory mechanisms controlling PP2A activity are not fully understood. In Sertoli cells, increase in PP2A activities under FSH conditions does not result from transcriptional up-regulation of PP2A-C isoforms, in agreement with the autoregulatory mechanism preventing fluctuation in PP2A-C translation (Baharians & Schonthal 1998). Evidence suggest that PP2A is mainly, if not exclusively, regulated by post-translational modifications of PP2A-C and regulatory subunits (Longin

*et al.* 2007), association of PP2A-C with regulatory proteins (Strack *et al.* 2004), and subcellular localization of the PP2A holoenzyme (Strack *et al.* 1998). However, timing of particulate PP2A activation in Sertoli cells seems exclude any acute post-translational modification of PP2A-C subunit, such as phosphorylation (Janssens & Goris 2001) and carboxymethylation (Longin *et al.* 2007) or phosphorylation of regulatory subunit of the holoenzyme (Ahn *et al.* 2007). A growing body of evidence suggests that a unique combination of regulatory subunits governs the enzymatic activity, substrate specificity, and location of PP2A enzymes (Janssens & Goris 2001, Strack *et al.* 2004, Janssens *et al.* 2005). Kinetics of particulate PP2A activity and protein expression in FSH-treated Sertoli cells suggest that a transcriptional mechanism is involved in the gonadotropin-induced up-regulation of particulate PP2A. The identification of the transcriptionally regulated B subunit involved in the recruitment/activation of particulate PP2A in Sertoli cells could be an interesting challenge for the future. Moreover, the inefficiency of both okadaic acid and calyculin to prevent the decrease in FSH-stimulated soluble PDE4 activities and the lack of FSH-induced up-regulation of soluble PP2A suggest that the mechanisms controlling the deactivation of PDE4 activities are different in particulate and soluble fractions. Whether differences are due to either the presence of structurally different PP2A holoenzymes or the involvement of different phosphatases in particulate and soluble fractions remains to be defined.

Whatever the nature of the mechanism triggered by FSH to target PP2A activities in the particulate fractions, it is clear that cell PGs, and more precisely transmembrane syndecans, play a role in this process. Simultaneous inhibition of both FSH-induced PP2A membrane expression and activation under chlorate conditions may suggest that syndecan-mediated anchorage of PP2A to the membrane is a prerequisite for PP2A activation, likely through association with other proteins in scaffolding complexes. Data on the signaling complexes associated with syndecans are limited, and to date only a still unidentified PP activated by fibroblast growth factor (FGF)-2 was found to be associated with the cytoplasmic tail of syndecan-4 through a PDZ adaptor protein (Horowitz *et al.* 2002). Due to the presence of an identical C-terminal PDZ-binding motif in all syndecans (Tkachenko *et al.* 2005), it is likely that such association is not restricted to syndecan-4. Moreover, the constitutive binding of regulatory subunits of PKA to the syndecan-1 cytoplasmic domain (Hayashida *et al.* 2006) suggests that PP2A and PKA could be closely anchored at the cell membrane, thereby facilitating PKA-mediated activation of particular PP2A holoenzyme recruited by syndecan-1 or another syndecan.

The temporally and spatially regulated expression of syndecans (Salmivirta & Jalkanen 1995, Tkachenko *et al.* 2005) suggests that these HSPGs fulfill developmental functions *in vivo*. Thus, it is noteworthy that cessation of Sertoli cell proliferation around 15 days *post partum* (Clermont & Pery 1957), a phenomenon associated in other cell types



with an increased expression of syndecan-1 (Leppa *et al.* 1992, Kato *et al.* 1995, Inki & Jalkanen 1996), is timely related to the ability of sodium chlorate to abolish particulate PDE4 deactivation. These data suggest that, in contrast with quiescent and differentiated Sertoli cells, PG-mediated particulate activation/recruitment of PP2A by FSH is not fully operational in proliferating Sertoli cells from 10-day-old rats albeit the maximal *in situ* immunoreactivity of PP2A-A subunit (van den Ham *et al.* 2003), whose postulated role is to stabilize the PP2A holoenzyme (Chen *et al.* 2005).

The promotion of interaction between PP2A and transmembrane PGs in non-proliferating Sertoli cells could likely fulfill more than a simple role in the reversal of the activated particulate PDE4D back to its pre-stimulatory, inactive state. Interestingly, the onset of fully operational PG-mediated recruitment/activation of particulate PP2A in response to FSH corresponds to the time of increase in cAMP pathway sensitization toward the gonadotropin (Levallet *et al.* 2007b). This period of transition from proliferating to differentiated state is also associated with switching of FSH receptor from MAPK3/1 (also known as Erk 1/2) activation to adenylate cyclase activation in Sertoli cells (Crepieux *et al.* 2001), a process requiring cell adhesion (Crepieux *et al.* 2002). Similar switching for the  $\beta_2$ -adrenergic receptor has been associated with dephosphorylation of the receptor (Daaka *et al.* 1997). Hence, syndecans, by their phenotype-dependent expression and their membrane location may fulfill yet unsuspected functions in the control of cellular responses to FSH or other external stimuli by anchoring and compartmentalize PP2A activity. Additional experiments will be required to identify the syndecan involved in these processes, even though the similar basolateral distribution of syndecan-1 (Miettinen *et al.* 1994) and FSH receptor (Vannier *et al.* 1996) suggest that syndecan-1 is the most probable candidate.

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