PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 68 (1999) 153–162

The Journal of Steroid Biochemistry & Molecular Biology

Role of proteoglycans on testosterone synthesis by purified Leydig cells from immature and mature rats

N. Grudet, P.J. Bonnamy, D. Le Goff, S. Carreau*

Laboratoire de Biochimie-IRBA, Université de Caen, Esplanade de la Paix, 14032 Caen Cedex, France

Received 22 June 1998; accepted 5 November 1998

Abstract

In order to characterize an involvement of proteoglycans (PG) in the regulation of Leydig cell function, we have examined the effects of *para*-nitrophenyl-β-D-xyloside (PNPX), a specific inhibitor of PG synthesis and *para*-nitrophenyl-β-D-galactoside (PNPG), an inefficient structural anologue, on testosterone production by purified Leydig cells from immature and mature rats, in the presence or not of various concentrations of hCG during 24 h. Whatever the age, the addition of PNPX induces a decrease of [^{35}S] and [^{3}H] incorporations into cell layer associated-PG; these latter being less numerous (−50 and −25%, respectively in immature and mature rat), and less sulfated (−40%) when compared to control Leydig cells. In immature Leydig cells, the inhibition of PG synthesis decreases both the basal and weakly stimulable-hCG or -$(Bu)_2$cAMP or -LH testosterone synthesis. In mature Leydig cells, the PG inhibition has no effect on testosterone production both in the absence of hCG and in the presence of weak amounts of hCG but increases it in the presence of subsaturating hCG concentrations. Whatever the age, the inhibition of PG synthesis is ineffective in the presence of saturating amounts of either hCG or $(Bu)_2$cAMP. These effects are maintained in the presence of MIX, PMA, but are not observed in the presence of 22*R*-hydroxycholesterol. Therefore, our results suggest that in rat Leydig cells, the inhibition of PG synthesis affects the signal transduction at a step distal to cyclic AMP and more precisely, the cholesterol supply to the mitochondria by acting on its cellular distribution (free and esterified cholesterol). © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Proteoglycans; Leydig cells; Steroidogenesis; Rat

1. Introduction

The mammalian testis is divided into two compartments, the seminiferous tubules with germ cells, peritubular cells and Sertoli cells which provide an architectural and nutritional supports for germ cells in development, and the interstitial tissue [1]. This latter, besides macrophages, contains Leydig cells which synthesize androgens required for the full development and the maintenance of spermatogenesis [2]. The regulation of immature and mature rat Leydig cell functions is not only controlled by LH, but also by intratesticular factors, among them growth factors acting as paracrine and/or autocrine factors [3–6]. In addition, it has become clear that the cell adhesion molecules are involved in Leydig cell functions such as migration, proliferation, differentiation and gene expression expression [7–11]. Proteoglycans (PG), which consist of a protein core containing at least one covalently bound glycosaminoglycan (GAG) [12,13], represent not only a family of extracellular matrix components but are also expressed on the cell surface [14–17]. PG participate in the cellular dynamic and regulation by their abilities to interact with either the extracellular matrix or membrane elements and by their capacities to bind growth factors [18–22]. By immunocytochemistry, Hayashi et al. [23] have shown the presence of PG in adult mouse Leydig cells. We have recently demonstrated that cultured immature and mature rat Leydig cells synthesize PG and heparan

* Corresponding author. Tel.: +33-2-315-65-488; fax: +33-2-315-65-320.

E-mail address: carreau@ibba.unicaen.fr (S. Carreau)

0960-0760/99/$ - see front matter © 1999 Elsevier Science Ltd. All rights reserved.
PII: S0960-0760(99)00027-8

sulfate proteoglycan (HSPG) [24]. Murono et al. [25], using heparin (a competitor of HSPG), suggest that in immature rat Leydig cells heparan sulfates are involved in the regulation of hCG binding by the basic fibroblast growth factor (bFGF), an intratesticular growth factor described as an heparin binding growth factor [26–28]. More recently, two studies using either sodium chlorate (an inhibitor of sulfation) or protamine sulfate and heparin have demonstrated that HSPG may play a role (i) in the immature rat Leydig cell production of androstane 3α,17β diol stimulated by bFGF only in the absence of LH [29] and (ii) in the mature rat Leydig cell, when high concentrations of LH [30] and bFGF [31] are used to stimulate the steroidogenesis.

In this report, we wondered to seek whether PG are implicated in the production of testosterone by immature and mature rat Leydig cells. For that purpose, we have used a specific inhibitor of PG synthesis, the *para*-nitrophenyl-β-D-xyloside (PNPX), an exogenous acceptor for galactosyl transferase I which competes with the endogenous xylosylated core protein for GAG chain elongation [32]. In parallel, to assess the specificity of the inhibition of PG synthesis, we have used a structural analogue the *para*-nitrophenyl-β-D-galactoside (PNPG) which does not alter the PG synthesis [33,34]. To understand the mechanism of action of PG at the cyclic AMP step, a cyclic analogue ($(Bu)_2$cAMP) and a specific inhibitor of phosphodiesterase, the 1-methyl-3-isobutyl-xanthine (MIX) [35] have been used. Moreover, subsequently to the action of LH/hCG, the protein kinase C is also activated [36]; so, a specific activator of protein kinase C, 4-β-phorbol-12-myristate-13-acetate (PMA) has been checked [37]. After the generation of cyclic AMP, the cholesterol is translocated from the cytosol to the mitochondrial membranes where it is converted into pregnenolone by the cholesterol side-chain cleavage complex (P450 scc). The true limiting step in the Leydig cell steroidogenesis is the delivery of the cholesterol to the inner mitochondrial membrane [38]. In order to sustain steroidogenesis and to determine if PG are implicated either in the cellular distribution or in the transport of cholesterol, we have first incubated Leydig cells with 22*R*-hydroxycholesterol (22*R*-OH cholesterol) [39] and secondly quantified both ester and free cholesterol.

2. Materials and methods

2.1. Reagents

Human chorionic gonadotropin (hCG) was from Organon (Serifontaine, France) and Ovine LH from NIDDKD-NIH (Bethesda, USA). DMEM-Ham F12 without sulfate was from Biological Industries (Kibbutz Beit Haemek, Israel). All other chemicals were purchased from Sigma (Saint-Quentin-Fallavier, France). Cationic membrane (Zeta-probe) was from Bio-Rad (Ivry-sur-Seine, France) and Percoll from Pharmacia Biotech AB (Uppsala, Sweden). [^{35}S]-sulfate and [^{3}H]-glucosamine were from NEN (Les Ulis, France) and [^{3}H]-testosterone from Amersham (Les Ulis, France).

2.2. Cell culture

Thirty day-old (immature) and 70/90 day-old (mature) male Sprague–Dawley rats from our own colony were killed by decapitation. The testes were decapsulated, submitted to an enzymatic dissociation, and the Leydig cells were purified (>80%) on Percoll gradients [40,41]. For the PG labeling experiments, Leydig cells (500,000 cells/cm^2) were seeded in 6-multiwell dishes (Falcon), then incubated for 24 h under an humidified atmosphere of 95% air/5% CO_2 at 32°C in 2 ml of sulfate-free medium containing [^{3}H]-glucosamine (2 μCi/ml) and [^{35}S]-sulfate (10 μCi/ml). Leydig cells were plated at a concentration of 200,000 cells/cm^2 in 24-multiwell dishes and cultured for 24 h in 1 ml of Ham F12-DMEM for determinations of testosterone and cholesterol. All cultures were conducted either in the presence or in the absence of PNPX, PNPG and various concentrations of LH/hCG. PNPX (1 mM), a concentration known to induce the biological effect [32–34], or PNPG (1 mM) was added to the purified Leydig cells and then, the production of testosterone was measured either in the absence or in the presence of hCG. PMA (100 ng/ml) and MIX (0.25 mM), concentrations which are not toxic for Leydig cells and biologically efficient [41,42], and 22*R*-OH cholesterol (30 μM), which allows a full stimulation of steroidogenesis [39], were used.

2.3. Quantitative solid-phase assay of ^{35}S- and ^{3}H-labeled macromolecules synthesized by immature and mature rat Leydig cells

At the end of the incubation, medium (2 ml) was collected and an equal volume of TUT 2X (100 mM Tris–HCl, 8 M urea, 1% Triton X-100, pH 7.2) containing protease inhibitors was added [33]. The cell layer was washed twice with PBS-Ca^{2+} (phosphate buffered saline 0.1 mM, Ca^{2+} 1 mM, pH 7.2). [^{35}S] and [^{3}H]-labeled materials associated with the cell layer (i.e. Leydig cells plus the putative extracellular matrix) were solubilized with 2 ml of TUT 2X under gentle shaking for 24 h at the room temperature. The well was rinsed with 2 ml of PBS-Ca^{2+} and pooled media (TUT 2X plus PBS-Ca^{2+}) were then brought to boiling. The quantifications of PG and GAG from the

Table 1
Effect of PNPX (1 mM) on proteoglycans synthesis by purified Leydig cells from (a) immature and (b) mature rats. Results, expressed in percentage, represent mean±SEM from three experiments

	[^{3}H]-Glucosamine		[^{35}S]-Sulfate	
	control	PNPX	control	PNPX
(a) Immature rats				
Secreted PG				
PG + GAG	100±3	223±26[a]	100±2.5	265±25[a]
PG	100±3	100±2.5[c]	100±7.5	86±4[c]
GAG	100±7.5	564±52[a]	100±12	1439±221[a]
Cell layer associated PG				
PG + GAG	100±4.5	60±4[a]	100±2	50±17[b]
PG	100±2.5	50±4.5[a]	100±3.5	34±3[a]
GAG	100±1.3	79±5[c]	100±19	138±30[c]
(b) Mature rats				
Secreted PG				
PG + GAG	100±6	116±12[c]	100±2	169±17[b]
PG	100±5	77±8[c]	100±1	96±8[c]
GAG	100±22	172±35[b]	100±5	269±32[a]
Cell layer associated PG				
PG + GAG	100±3	64±6[a]	100±3	54±6[a]
PG	100±6	75±5[b]	100±4	54±2[a]
GAG	100±13	27±4[a]	100±8	56±14[b]

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] Not significant.

medium and cell layer extracts were determined by solid phase assay using cationic nylon membrane [43] sandwiched in a 96-well dot blot apparatus (Bio-Rad). PG and GAG were quantified using our reported methods [24,44]. For each culture well, radioactivity incorporated in free GAG chains was determined by subtracting the radioactivity of the 0.15 M NaCl-treated dot from that of the 0.9 M NaCl-treated dot [34,44].

2.4. Measurements of testosterone and cholesterol

The culture medium containing more than 90% of the testosterone synthesized by the Leydig cells [41] was removed and testosterone determined by RIA using a specific antibody purchased from BioMerieux (Lyon, France). The inter- and intra-assay coefficients of variations were respectively 3 and 6%, and the sensitivity 4 pg/tube. The cell lipids were extracted by the procedure of Hara and Radin [45] after washings of the Leydig cells with cold BSA-PBS (0.1%). Free and total cholesterol were measured enzymatically as described by Salè et al. [46]; cholesterol esters were determined after hydrolysis, by subtracting the values of free from total cholesterol.

2.5. Statistical analysis

All experimental data are presented as means±SEM of duplicate determinations from three wells in three different experiments. The Student's *t*-test was used to compare the mean values and statistical significance was accepted at $p < 0.05$ (StatWorks, Brain Power, Calabasas, CA).

3. Results

3.1. Effect of PNPX on PG and GAG synthesized by rat Leydig cells

Whatever the treatment, after 24 h of culture, the Leydig cell viability was 90% and the percentage of 3β-hydroxysteroid dehydrogenase positive cells was around 70%.

3.1.1. Immature rat Leydig cells

The addition of PNPX to the Leydig cell cultures did not modify the [^{3}H]-glucosamine incorporation in secreted PG but induced a significant decrease in newly synthesized [^{3}H]-PG associated with the cell

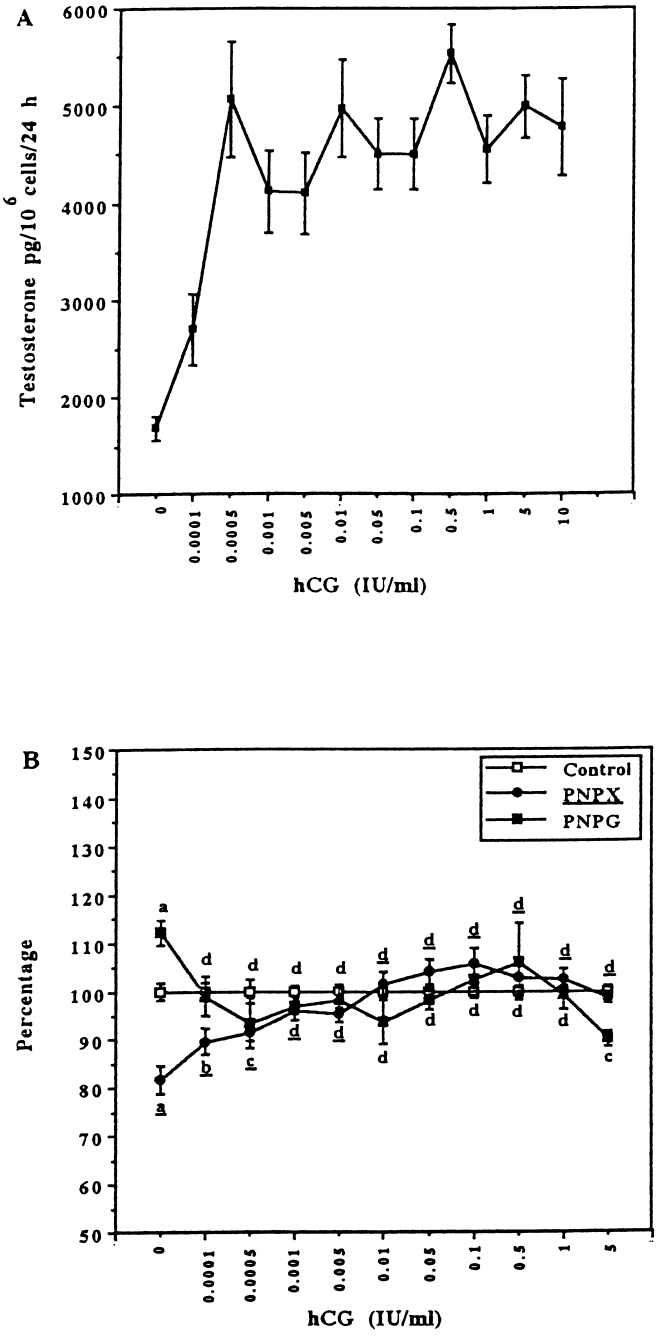

Fig. 1. (A) Testosterone synthesis by immature rat Leydig cells incubated 24 h in the absence and in the presence of increasing concentrations of hCG. Results, expressed in pg/10^6 Leydig cells/24 h, represent means±SEM of at least four independent experiments. (B) Effects of PNPX (1 mM) and of PNPG (1 mM) on the response of immature rat Leydig cells to hCG. Leydig cells were incubated for 24 h with increasing concentrations of hCG in the absence and in the presence of PNPX or PNPG. Data, expressed in percentage, represent means±SEM of at least four experiments. (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$; (d) not significant when we compared testosterone production in the absence versus in the presence of either PNPX (underlined) or PNPG.

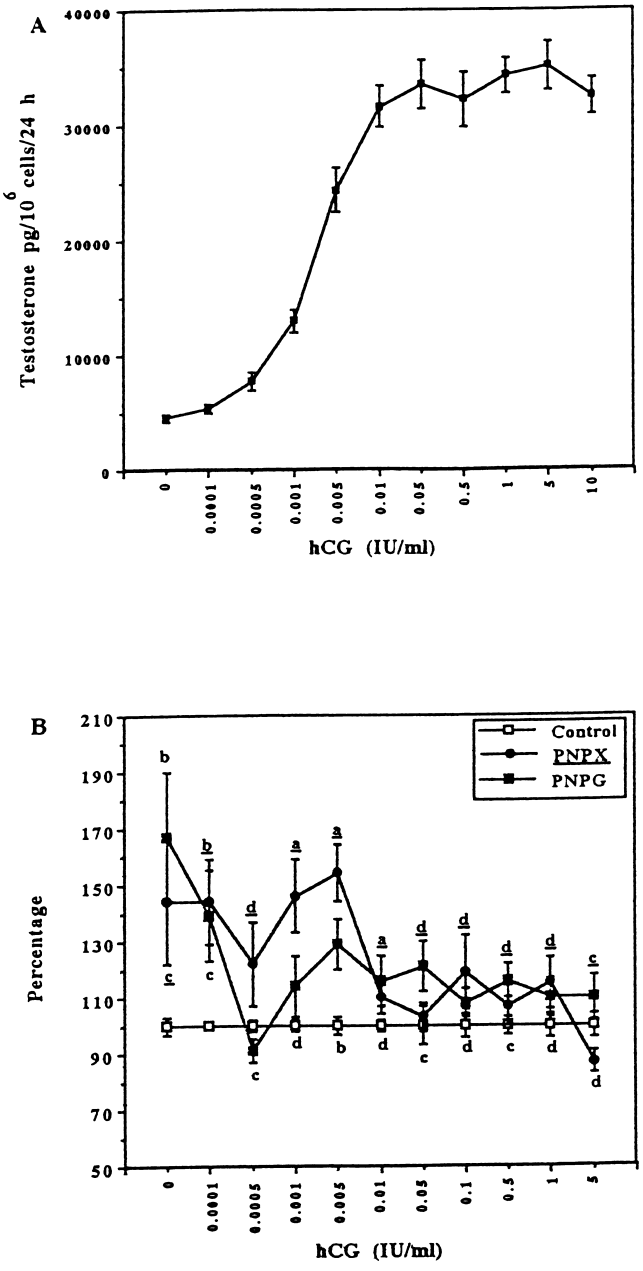

Fig. 2. (A) Testosterone synthesis by mature rat Leydig cells after 24 h of incubation in the absence and in the presence of increasing concentrations of hCG. Results, expressed in pg/10^6 Leydig cells/24 h, represent means±SEM of at least four separated experiments. (B) Effects of PNPX (1 mM) and PNPG (1 mM) on the response of mature rat Leydig cells to hCG. Leydig cells were incubated for 24 h with increasing concentrations of hCG in the absence or in the presence of PNPX or PNPG. Results, expressed in percentage, represent means±SEM of at least four experiments. (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$; (d) not significant for comparisons of testosterone production in the absence and in the presence of either PNPX (underlined) or PNPG.

layer. Simultaneously, PNPX induced a great increase in the secretion of [^{3}H]-GAG whereas no significant effect on [^{3}H]-glucosamine incorporation in the cell layer associated GAG was recorded. Furthermore, PNPX had no significant effect on the incorporation of [^{35}S] in the secreted PG but induced a large decrease in [^{35}S]-PG associated with the cell layer. Concerning the secreted GAG, PNPX provoked a drastic augmentation in [^{35}S]-sulfate incorporation but was ineffective on the [^{35}S]-labeling of the cell layer GAG (Table 1a).

3.1.2. Mature rat Leydig cells

The PNPX induced a significant decrease in newly synthesized [^{3}H]-PG secreted and associated with the cell layer. Simultaneously, PNPX enhanced the secretion of GAG but, in contrast, had a negative effect on [^{3}H]-glucosamine incorporation in the cell layer associated GAG. Moreover PNPX was ineffective on the synthesis of newly synthesized [^{35}S]-secreted PG but induced a significant decrease the incorporation of [^{35}S] in PG associated with the cell layer. PNPX caused an increase in [^{35}S]-secreted GAG and a diminution of [^{35}S]-sulfate incorporation in the cell layer associated-GAG (Table 1b).

3.2. Effect of PNPX on hCG-stimulated testosterone production

3.2.1. Immature Leydig cells

Purified Leydig cells from immature rats cultured for 24 h in the absence of hCG (or LH) produced 1.7$\pm$0.1 ng of testosterone/10^6 Leydig cells. This synthesis was stimulated weakly by hCG at 10^{-4} IU/ml ($\times$1.6), whereas a 2.8-fold increase of testosterone output was obtained for higher concentrations (Fig. 1A). Similarly, the testosterone production was slightly enhanced (1.2-fold) by low amounts of $(Bu)_2$cAMP (10^{-4}–10^{-2} mM) whereas 1.4- and 2.5-fold increases of testosterone output were recorded for 0.1 and 1 mM of $(Bu)_2$cAMP (data not shown).

The addition of PNPX to Leydig cell cultures induced significant decreases in testosterone production in the absence (−18%) as well as in the presence of either low concentrations of hCG (−10%) or $(Bu)_2$cAMP (−20%). In contrast, in the presence of higher amounts of either hCG or $(Bu)_2$cAMP, PNPX was ineffective (Fig. 1B). These observations were confirmed when different concentrations of LH were used instead of hCG (data not shown).

3.2.2. Mature Leydig cells

After 24 h, the basal production of testosterone was 4.5$\pm$0.3 ng/10^6 Leydig cells. This synthesis was weakly enhanced (1.2-fold) by 10^{-4} IU/ml of hCG; conversely, the testosterone output was increased 7.3-fold when saturating amounts of hCG (from 10^{-2} to 5 IU/ml) were used (Fig. 2A). In the presence of $(Bu)_2$cAMP, the testosterone secretion was increased in a dose-related manner and reached 27.5 ng/10^6 cells/24 h (data not shown).

In the presence of PNPX the testosterone production was significantly increased both in the absence (+44%) and in the presence (+41%) of weakly stimulating amounts of hCG. However, the synthesis of testosterone was not modified by PNPX when saturating concentrations of hCG were used whereas it was significantly reduced in the presence of 5 IU/ml of hCG (Fig. 2B). When Leydig cells were cultured with PNPX and low amounts of $(Bu)_2$cAMP a significant effect was recorded on the testosterone output (+50%) whereas for higher concentrations, PNPX was less effective (+20%) (data not shown).

3.3. PNPX effect on hCG-stimulated testosterone production: is it related to inhibition of PG synthesis?

3.3.1. Immature Leydig cells

The addition of PNPG induced a slight increase (+12%) of testosterone production in the absence of hCG whereas in the presence of subsaturating or saturating hCG concentration the production of testosterone was unchanged (Fig. 1B). Similar effects were observed when $(Bu)_2$cAMP was used (data not shown). The effects of PNPX and PNPG were significantly different in the absence of treatment and in the presence of subsaturating stimulation; consequently, PG inhibition decreased testosterone synthesis respectively by 30% ($p < 0.001$) and by 10% ($p < 0.05$).

3.3.2. Mature Leydig cells

In the presence of PNPG, the testosterone production was enhanced in the absence (+65%) and in the presence of low amounts of hCG (+40%). When PNPG was added together with higher concentrations of hCG, the production of testosterone was slightly increased (+13%, Fig. 2B). When low concentrations of $(Bu)_2$cAMP were used PNPG was not efficient whereas for higher amounts, PNPG induced an increase of testosterone outputs (data shown). The effects of PNPX and PNPG were significantly different in the presence of subsaturating stimulation; consequently, PG inhibition increased the testosterone synthesis by 30% ($p < 0.05$).

3.4. Signal transduction step(s) affected by the inhibition of PG synthesis

3.4.1. Effect of PMA and MIX

Since the effect of PG inhibition was mainly observed in the absence or in the presence of low hCG concentrations, we have investigated the potential role of PG on testosterone production by immature and

mature Leydig cells incubated in the presence of MIX (0.25 mM) and PMA (100 ng/ml).

In the absence of treatment, PNPX and MIX alone had two opposite and significant effects on testosterone secretion by immature Leydig cells; in the presence of both PNPX and MIX, the testosterone production was not different from that obtained without treatment. To assess the specificity of the PG inhibition, PNPG was used and as reported above, PNPG increased significantly the testosterone production and its effect was different from that with PNPX. In the presence of MIX and PNPG, the testosterone production was further increased (+61%, $p < 0.001$, Fig. 3A). Moreover as observed in the absence of MIX, the effects of PNPG-MIX and PNPX-MIX were significantly different. Therefore, it appeared that the PG inhibitory effect on testosterone production by immature Leydig cells was recorded in the absence (−55%, $p < 0.001$) and in the presence of MIX (−50%, $p < 0.001$, Fig. 3A). These observations were observed also in the presence of a low hCG concentration (data not shown).

Although the effect of PG inhibition was not significant on testosterone production by mature Leydig cells in the absence of treatment, we have cultured the Leydig cells in the presence of MIX. As for immature Leydig cells, the effects of PNPX-MIX and PNPG-MIX were equivalent to the sum of their separated ones (Fig. 4).

After the incubation of immature Leydig cells for 24 h in the presence of PMA, an increase of testosterone secretion was noticed and the effect was significantly different from that obtained with PNPX which decreased testosterone secretion. However, in the presence of both PMA and PNPX, the testosterone secretion was not different from that without treatment (Fig. 3B).

3.4.2. Role of cholesterol supply

In order to evaluate a potential effect of PG on the cholesterol delivery to the mitochondria, the Leydig cells were incubated with 22*R*-OH cholesterol (30 μM) for 24 h and with either PNPX or PNPG.

3.4.2.1. Immature Leydig cells. In the presence of 22*R*-OH cholesterol, the basal testosterone production by immature Leydig cells was tremendously enhanced (34-fold). In the presence of 22*R*-OH cholesterol and of PG synthesis inhibition, the testosterone production was not significantly increased in the absence of hCG. Furthermore, in the absence of treatment, the cholesterol esters were largely predominant (89% of the total cholesterol) in immature Leydig cells (Table 2a). The inhibition of PG synthesis and sulfation did not affect the quantity of total cholesterol but modified its cellular distribution; in the presence of PG inhibition, the free cholesterol was decreased and conversely, the quantity of cholesterol esters was enhanced (+24%, $p < 0.01$, Table 2b).

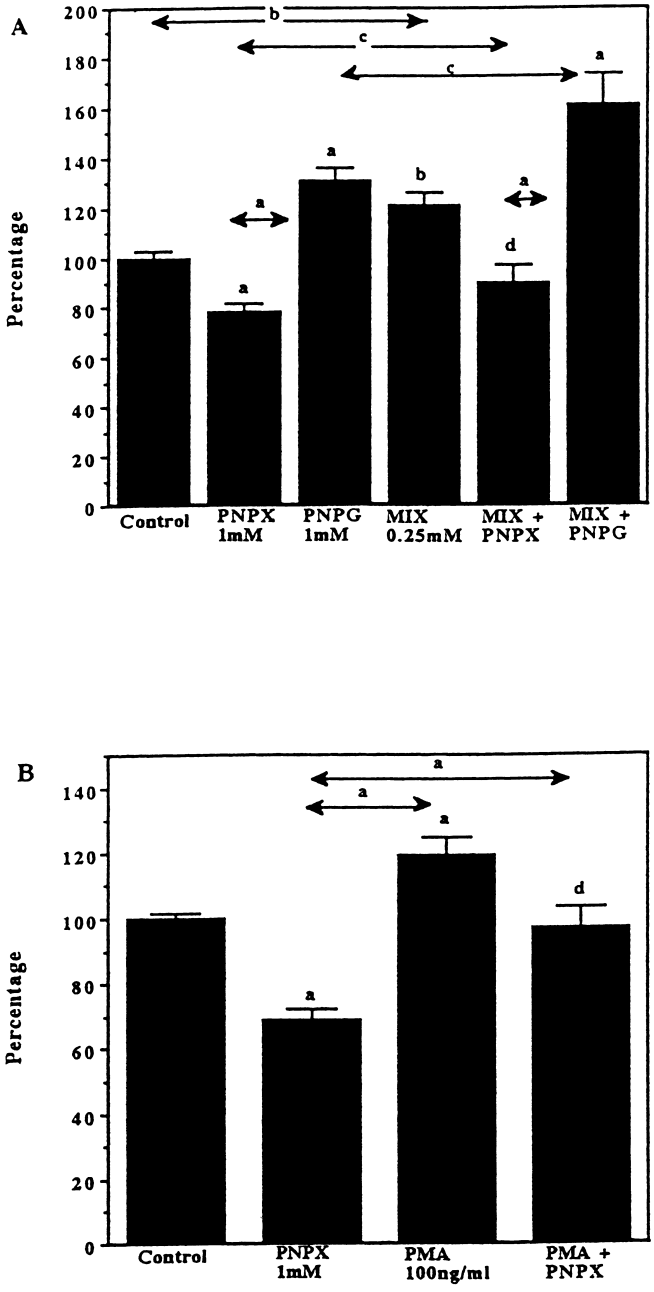

Fig. 3. (A) Testosterone synthesis by immature rat Leydig cells incubated 24 h in the absence of hCG and in the presence or not of MIX (0.25 mM), PNPX (1 mM), PNPG (1 mM). Results, expressed in percentage, represent means±SEM of two different experiments. (B) Testosterone synthesis by immature rat Leydig cells incubated 24 h in the absence of hCG and in the presence or not of PMA (100 ng/ml), PNPX (1 mM). Results, expressed in percentage, represent means±SEM of two different experiments. (a) $p < 0.001$; (b) $p < 0.01$; (c) $p < 0.05$; (d) not significant when we compared testosterone production recorded in the absence and in the presence of either PNPX or PNPG.

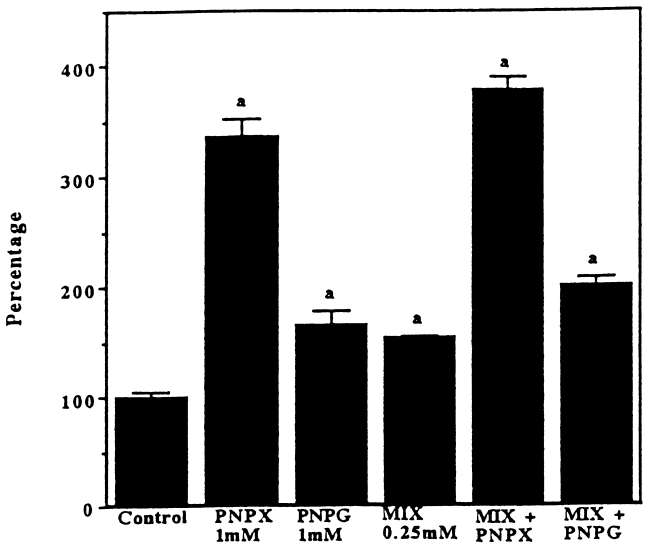

Fig. 4. Testosterone synthesis by mature rat Leydig cells incubated 24 h in the absence of hCG and in the presence or not of MIX (0.25 mM), PNPX (1 mM), PNPG (1 mM). Results, expressed in percentage, represent means±SEM of three wells from one experiment. (a) $p < 0.001$, when we compared of testosterone productions without and with treatment.

3.4.2.2. Mature Leydig cells. In the presence of 22*R*-OH cholesterol, the basal synthesis of testosterone by mature Leydig cells was increased 15-fold. In the presence of 22*R*-OH cholesterol and in the absence of hCG, PNPX and PNPG effects on testosterone production were not significantly different; therefore, the PG inhibition was ineffective on testosterone production by mature Leydig cells. In the absence of treatment, the free cholesterol was much less important than the cholesterol esters, representing respectively 21 and 79% of the total cholesterol recovered from mature Leydig cells (Table 2a).

When either PNPX or PNPG was added, the quantity of total cholesterol was unchanged but the cellular distribution of cholesterol was affected. In the presence of PNPX, whereas cholesterol esters were decreased the free cholesterol was enhanced. On the contrary, in the presence of PNPG, the free cholesterol was decreased and cholesterol esters were increased. Thus the PG inhibition, without affecting the quantity of total cholesterol, by increasing the free cholesterol and decreasing the cholesterol esters (−34%), modified the cellular distribution of cholesterol (Table 2b).

4. Discussion

In a previous work, we have demonstrated a developmental regulation of PG associated with the cell layer synthesis; these latters and HSPG are more numerous in immature than in mature rat Leydig cells [24]. Herein, we used a specific inhibitor of PG synthesis, PNPX, to examine the effects of PG sulfation and synthesis on the testosterone secretion by immature and mature rat Leydig cells cultured for 24 h in the presence or not of various concentrations of either hCG, or LH or $(Bu)_2$cAMP.

Whatever the age, PNPX induces both quantitative and qualitative modifications of PG synthesis in rat Leydig cells. Indeed, PG associated with the cell layer produced by immature and mature rat Leydig cells are not only quantitatively decreased but also less sulfated. As previously shown with other cell types, no heparan sulfate is formed on xylosid primer but the HS synthesis is diminished due to a decrease of precursors availability for PNPX-GAG synthesis [32–34].

Both in immature and mature Leydig cells the testosterone secretion is modified in the presence of PNPG, an inefficient competitor of PG synthesis, therefore the true effect of PG inhibition is not obvious. This observation and the fact that PNPX can be catabolized (giving paranitrophenol and xylose) in different cell types [47,48] suggest that the aglycone el-

Table 2
(a) Quantification of total, free and ester of cholesterol and (b) effect of PNPX (1 mM), PNPG (1 mM) and PG inhibition on free cholesterol in immature and mature rat Leydig cells cultured for 24 h in the absence of hCG. Results represent mean±SEM from three and two experiments respectively in immature and mature rat Leydig cells

	Total cholesterol (μg/10^6 cells)	Free cholesterol (μg/10^6 cells)	Ester of cholesterol (μg/10^6 cells)
Immature rat Leydig cells	18.2±0.6	2.1±0.8	16.8±1.8
Mature rat Leydig cells	11.8±1.8	2.4±0.1	9.4±0.2

	Total cholesterol (μg/10^6 cells)	Free cholesterol (%)		PG inhibition effect
		control	PNPX	PNPG
Immature rat Leydig cells	18.2±0.6	11.1±0.5	21.3±2	43±5 −50%[a]
Mature rat Leydig cells	11.8±1.8	20±1.1	40.5±0.5	14±1.1 +133%[a]

[a] $P < 0.01$.

ement of PNPX and PNPG is efficient on testosterone secretion. Consequently we have subtracted the effect observed in the presence of PNPG from that obtained with PNPX to determine the real effect of PG inhibition on testosterone production.

In the absence of gonadotropin and in the presence of both sulfation and synthesis inhibitions of PG, the testosterone secretion is significantly decreased in immature but not significantly modified in mature Leydig cells. Our results are identical to those reported when only the inhibition of PG sulfation is induced in immature [31] and in mature [30] rat Leydig cells. Therefore, we can exclude the hypothesis that biological effects of PG upon cultured Leydig cells can be due to an alteration of membrane conformation subsequent to inhibition of PG synthesis. Concerning the effect of PG inhibition, we have supposed an involvement of cAMP since PG inhibition is effective in unstimulating and subsaturating hCG (or LH or $(Bu)_2$cAMP) conditions. Therefore, we have studied the effects of PMA and MIX on testosterone production in immature and mature rat Leydig cells in the presence of either PNPX or PNPG and without stimulation of testosterone secretion. As shown in a previous study [49], the addition of PMA as well as that of MIX to the Leydig cell incubation medium, increases the testosterone secretion [2,50]. Since in the absence or presence of low hCG concentrations, the effects obtained with either PNPX-MIX or PNPG-MIX are the sum of their separated effects, we conclude that whatever the role of PNPX or PNPG or PG inhibition, their effects are maintained in the presence of MIX. Similar observations are recorded in the presence of PMA. Therefore, both in immature and mature rats, the effects of PNPX and PNPG in the absence of treatment are located at a post-cyclic AMP step. These results are observed also using subsaturating and unstimulating concentrations of hCG, respectively in immature and mature rat Leydig cells. Whatever the age, in the absence of hCG but in the presence of 22*R*-OH cholesterol, the PG inhibition is inefficient on the testosterone secretion. Since the decrease induced by the PG inhibition is maintained in the presence of both PMA, MIX or very low amounts of hCG (or LH or $(Bu)_2$cAMP) and is abolished in the presence of 22*R*-OH cholesterol, PG appear to be essential for the maintenance of cholesterol supply.

Consequently, we may suggest that in immature rat Leydig cells, both in the absence or in the presence of low concentrations of hCG (or LH or $(Bu)_2$cAMP), an autocrine positive factor may interact with PG, and more precisely with their GAG chains, in order to maintain the delivery of cholesterol to the mitochondrial P450 scc. Since the PG inhibition, without affecting the quantity of cholesterol, modifies the cellular distribution of cholesterol, by increasing cholesterol esters and decreasing free cholesterol, we may evoke a positive role for PG in activity and/or synthesis of ester hydrolases in immature rat Leydig cells.

Conversely, in mature rat Leydig cells, the PG inhibition is inefficient in the absence as well as in the presence of unstimulating concentrations of hCG, but increases the testosterone production in the presence of subsaturating doses of gonadotropin. However, as in immature rat Leydig cells, the PG inhibition effect disappears or is not predominant in the presence of either high concentrations of hCG or 22*R*-OH cholesterol. So, in mature Leydig cells, we may suggest the implication of an autocrine negative factor which action requires the interaction with PG and more precisely, with their GAG chains. Since its action is post-cAMP and is suppressed in the presence of 22*R*-OH cholesterol, that factor acts on the delivery of cholesterol to the steroidogenic pathway stimulated by hCG. Indeed, PG inhibition increases the disponibility of free cholesterol (two-fold) by decreasing cholesterol esters. So we may postulate the presence of an autocrine negative factor, which requires PG and thus limits activity and/or synthesis of ester hydrolases.

So whatever the age, in the presence of a saturating concentration of either hCG or $(Bu)_2$cAMP or LH, PG are inefficient in improving testosterone output. So, our results are opposite to those obtained in previous studies [30,31]. Our observations cannot be explained by the fact that hCG modulates the cell surface PG species and/or their abundance. Even if hCG modifies the PG synthesis by Leydig cells, as shown in our previous study [24], these latters are secreted PNPX-GAG. Our data and those of McFarlanne et al. [30] and Laslett et al. [31] observed in the presence of high LH concentration are difficult to reconcile. In mature Leydig cells, protamine sulfate and sodium chlorate induce a decrease by more 50% of the testosterone secretion in the presence of a saturating amount of LH. However, after removing the culture medium, there is no difference between the production of testosterone by preincubated Leydig cells with either sodium chlorate or not during 20 h, followed by the addition of a saturating amount of LH [30]. These observations and our results suggest (i) that PG have no effect in the presence of high LH/hCG concentrations and (ii) that the effect observed by McFarlanne et al. [30] with sodium chlorate does not implicate PG and/or GAG chains. Thus that drug which is a sulfation inhibitor non-specific of PG or GAG may interfere with other molecules involved in the regulation of testosterone secretion by both immature and mature rat Leydig cells.

In conclusion we have demonstrated that PG and HSPG associated with the cell layer are quantitatively more important in immature than in mature rat Leydig cells [24]. This age-related developmental difference in PG synthesis is in agreement (i) with the exist-

ence of a basement membrane [8] and (ii) with their role in the modulation of steroidogenesis in immature and mature rat Leydig cells.

Acknowledgements

The authors wish to thank the NIDDK (NIH, Bethesda, MD, USA) for the gift of ovine LH and Mrs. Edine for her technical assistance in rat testicular cell preparations.

References

[1] M.K. Skinner, Cell–cell interactions in the testis, Endocrine Rev. 15 (1991) 574–606.
[2] J.M. Saez, Leydig cells: endocrine, paracrine and autocrine regulation, Endocrine Rev. 15 (1994) 574–606.
[3] S. Carreau, Germ cells–Sertoli cells interactions and Leydig cells function, in: M.L. Dufau, A. Fabbri, A. Isidori (Eds.), Cell and Molecular Biology of the Testis, Serono Symposia Publications, Frontiers Endocrinol, 5 (1994) 137–148.
[4] M. Benhamed, Growth factors and cytokines in the testis, in: F.H. Combaire (Ed.), Male infertility, vol. 4, Chapman and Hall, 1995, pp. 56–94.
[5] J.M. Saez, H. Lejeune, O. Avallet, R. Habert, P. Durand, Le contrôle des fonctions differenciées des cellules de Leydig, Medecine/Sciences 11 (1995) 547–553.
[6] H. Lejeune, B. Jegou, S. Carreau, J.M. Saez, Régulation paracrine et autocrine des fonctions testiculaires, in: J. Belaish, M.A. Drosdowsky, A. Vermeulen (Eds.), L'Endocrinologie Sexuelle de l'Homme, vol. 7, Doin, Paris, 1996, pp. 77–103.
[7] R.B. Vernon, T.M. Lane, J.C. Angello, H. Sage, Adhesion, shape, proliferation and gene expression of mouse Leydig cells are influenced by extracellular matrix in vitro, Biol. Reprod. 44 (1991) 157–170.
[8] I. Huhtaniemi, L.J. Pelliniemi, Fetal Leydig cells: cellular origin, morphology, life span, and special functionnal features, PSEBM 201 (1992) 125–140.
[9] A.M. Mayerhofer, G. Lahr, K.L. Seidl, B. Eusterschulte, A. Christoph, M. Gratzl, The neural cell adhesion molecule (NCAM) provides clues of the development of testicular Leydig cells, J. Androl. 17 (1996) 223–230.
[10] L.J. Pelliniemi, T. Kuoppio, K. Frödjman, The cell biology and function of the fetal Leydig cell, in: A.H. Payne, M.P. Hardy, L.D. Russell (Eds.), The Leydig Cell, Cache River Press, FL, 1996, pp. 143–157 chap. 5.
[11] N. Wu, E.P. Murono, W.E. Caruer, L. Terracio, T. Bacro, Evidence that α5β1 integrins mediate Leydig cell binding to fibronectin and enhance Leydig cell proliferation stimulated by a Sertoli cell-secreted mitogenic factor in vitro, Endocrine 5 (1996) 75–83.
[12] L.A. Ruoslathi, Structure and biology of proteoglycans, Ann. Rev. Cell. Biol. 4 (1988) 229–255.
[13] T.E. Hardingham, A.J. Fosang, Proteoglycans: many forms and many functions, FASEB 6 (1992) 861–871.
[14] J.T. Gallagher, The extented family of proteoglycans: social residents of the pericellular zone, Curr. Opin. Cell. Biol. 1 (1989) 1201–1218.
[15] L. Kjellen, U. Lindahl, Proteoglycans: structures and interactions, Ann. Rev. Biochem. 60 (1991) 443–475.
[16] T.M. Wight, D.K. Heinegard, V.C. Hascall, Proteoglycans: structure and function, in: E.D. Hay (Ed.), Cell of Biology of Extracellular Matrix, Plenum Press, New York, 1991, pp. 45–78.
[17] J.R. Couchman, A. Woods, Structure and biology of pericellular proteoglycans, in: D.D. Roberts, R.P. Mecham (Eds.), Cell suface and extracellular glycoconjugates, Academic Press, San Diego, 1993, pp. 33–79.
[18] M. Bernfield, R. Kokenyesi, M. Kato, M.T. Hinkes, R.L. Gallo, E.J. Lose, Biology of syndecans: a family of transmembrane heparan sulfate proteoglycans, Ann. Rev. Cell. Biol. 8 (1992) 365–393.
[19] D.M. Templeton, Proteoglycans in cell regulation, Crit. Rev. Clin. Lab. Sci. 29 (1992) 141–184.
[20] T.M. Wight, M.G. Kinsella, E.E. Qwarnstom, The role of proteoglycans in cell adhesion, migration and proliferation, Curr. Op. Cell. Biol. 4 (1992) 793–801.
[21] G. David, Integral membrane heparan sulfate proteoglycans, FASEB 7 (1993) 1023–1030.
[22] J.R. Couchman, A. Woods, Syndecans, signalling, and cell adhesion, J. Cell. Biochem. 61 (1996) 578–584.
[23] K. Hayashi, M. Hayashi, M. Jalkanen, J.H. Firestone, R.L. Trelstad, M. Bernfield, Immunocytochemistry of cell surface heparan sulfate proteoglycan in mouse tissues. A light and electron microscopy study, J. Histochem. Cytochem. 35 (1987) 1078–1088.
[24] N. Grudet, P.J. Bonnamy, J. Bocquet, S. Carreau, Proteoglycan synthesis by purified Leydig cells from immature and mature rats, C.R. Acad. Sci. Paris 319 (1996) 1101–1105.
[25] E.P. Murono, A.L. Washburn, D.B. Goforth, N. Wu, Evidence that both receptor- and heparan sulfate proteoglycan-bound basic fibroblast growth factor are internalysed by cultured immature Leydig cells, Mol. Cell. Endocrinol. 98 (1993) 81–90.
[26] N. Quarto, F. Amalric, Heparan sulfate proteoglycans as transducers of FGF-2 signalling, J. Cell. Sci. 107 (1994) 3201–3212.
[27] H.Q. Miao, T.A. Fritz, D.J. Esko, J. Zimmermann, A. Yayon, I. Vlodavsky, Heparan sulfate primed on β D-xylosides restores binding of basic fibroblast growth factor, J. Cell. Biochem. 57 (1995) 173–184.
[28] R.G. Gambarini, M.T.M. Miranda, W. Viviani, S. Oyama Jr., S. Kiyota, N. Toma, Structure and function of fibroblast growth factor, Brazilian J. Med. Biol. Res. 29 (1996) 835–839.
[29] A.L. Laslett, J.R. McFarlanne, G.P. Risbridger, Developmental response by Leydig cells to acidic and basic fibroblast growth factors, J. Steroid Biochem. Molec. Biol. 60 (1997) 171–179.
[30] J.R. Mc Farlanne, A.L. Laslett, G.P. Risbridger, Evidence that heparin binding autocrine factors modulates testosterone production by the adult rat Leydig cell, Mol. Cell. Endocrinol. 118 (1996) 57–63.
[31] A.L. Laslett, J.R. Mc Farlanne, M.T.W. Hearn, G.P. Risbridger, Requirement for heparan sulfate proteoglycans to mediate basic fibroblast growth factor (FGF2)-induced stimulation of Leydig cell steroidogenesis, J. Steroid Biochem. Molec. Biol. 4 (1995) 245–250.
[32] R.K. Margolis, B. Gossen, H. Tekotte, L. Hilgenberg, R.U. Margolis, Effects of β-xylosides on proteoglycan biosynthesis and morphology of PC 12 pheochromocitoma cells and primary cultures of rat cerebellum, J. Cell. Sci. 99 (1991) 237–246.
[33] D.J. Carey, Biological functions of proteoglycans: use of specific inhibitors of proteoglycan synthesis, Mol. Cell. Biochem. 104 (1991) 21–28.
[34] N.T. Phamanthu, P.J. Bonnamy, M. Bouakka, J. Bocquet, Inhibition of proteoglycan synthesis induces an increase in follicle stimulating hormone (FSH)-stimulated estradiol production by immature rat Sertoli cells, Mol. Cell. Endocrinol. 109 (1995) 37–45.
[35] M. Conti, G. Nemoz, C. Sette, E. Vicini, Recent progress in

understanding the hormonal regulation of phosphodiesterases, Endocrine Rev. 16 (1995) 370–389.

[36] P.C.K. Leung, G.L. Steele, Intracellular signaling in the gonads, Endocrine Rev. 13 (1992) 476–498.

[37] A.C. Newton, Regulation of protein kinase C, Curr. Opin. Cell. Biol. 9 (1997) 161–167.

[38] D.M. Stocco, B.J. Lark, Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis, Biochem. Pharmacol. 51 (1996) 197–205.

[39] V. Papadopoulos, P. Kamtchouing, M.A. Drosdowsky, M.T. Hochereau de Reviers, S. Carreau, Adult Sertoli cells secrete a factor or factors which modulate Leydig cell function, J. Endocrinol. 114 (1987) 459–467.

[40] A. Lefevre, J.M. Saez, L. Finaz, hCG responsiveness of purified Leydig cells in immature and mature rats, Hormone Res. 17 (1983) 114–120.

[41] V. Papadopoulos, S. Carreau, M.A. Drosdowsky, Effect of phorbol ester and phospholipase C on LH-stimulated steroidogenesis in purified rat Leydig cells, FEBS Lett. 188 (1985) 312–316.

[42] J.U. Wurthner, M. Kistler, M. Kratzmeier, A.K. Mukhopadhyay, LH/hCG receptor is coupled to both adenylate cyclase and protein kinase C signalling pathways in isolated mouse Leydig cell, Endocrine 3 (1995) 579–584.

[43] A. Rappraeger, C. Yeaman, A quantitative solid-phase assay for identifying radiolabeled glycosaminoglycans in crude cell extract, Anal. Biochem. 179 (1989) 361–365.

[44] B. Thièbot, L. Bichoualne, M. Langris, P.J. Bonnamy, P. Barbey, S. Carreau, J. Bocquet, IGF-1 stimulates synthesis of undersulfated proteoglycans and of hyaluronic acid by peritubular cells from immature rat testis, Biochim. Biophys. Acta 1358 (1997) 127–141.

[45] A. Hara, N.S. Radin, Lipid extraction of tissues with a low-toxicity solvent, Anal. Biochem. 90 (1978) 420–426.

[46] F.O. Salè, S. Marchesini, P.H. Fishman, B. Berra, A sensitive enzymatic assay for determination of cholesterol in lipid extracts, Anal. Biochem. 48 (1984) 347–350.

[47] M.C. Farrach, J.Y. Ping Tang, G.L. Decker, D.D. Carson, Differential effect of *p*-nitrophenyl-D-xylosides on mouse blastocysts and uterine epithelial cells, Biol. Reprod. 39 (1988) 443–455.

[48] S. Potter-Perigo, K.R. Braun, E. Schonherr, T.N. Wight, Altered proteoglycan synthesis via the false acceptor pathway can be dissociated from β-xyloside inhibition of proliferation, Archiv. Biochem. Biophys. 297 (1992) 101–109.

[49] H. Nikula, I. Huhtaniemi, Effect of protein kinase C activation on cyclic AMP and testosterone production of rat Leydig cells in vitro, Acta Endocrinol. 121 (1989) 327–333.

[50] B.A. Cooke, M.C.K. Choi, G. Dirami, M.P. Lopez-Ruiz, A.P. West, Control of steroidogenesis in Leydig cells, J. Steroid Biochem. Molec. Biol. 43 (1992) 445–449.