Journal of Cellular Biochemistry 76:322–331 (1999)

Inhibition of Transmembrane Calcium Influx Induces Decrease in Proteoglycan Synthesis in Immature Rat Sertoli Cells

Gaëlle Fagnen,* Nhu Thu Phamantu, Jean Bocquet, and Pierre-Jacques Bonnamy

Laboratoire de Biochimie, UPRES-EA 2608, I.R.B.A., Université de Caen, Esplanade de la Paix, 14032 Caen Cedex, France

Abstract Beyond increased cAMP synthesis, calcium influx has been involved in signal transduction triggered by the gonadotropin follicle-stimulating hormone (FSH), the main regulator of Sertoli cells functions. In order to delineate a possible involvement of calcium in the regulation of proteoglycan synthesis, we have examined the effect of low-voltage-activated calcium channel blocker verapamil on both [^{35}S]-sulfate and [^{3}H]-glucosamine incorporation into proteoglycan molecules neosynthesized by cultured Sertoli cells from 20-day-old rats. Verapamil induced a dose- and time-dependent decrease in labeling of both secreted and cell-associated proteoglycans, as determined by quantitative solid-phase assay. This effect was mimicked by the addition of the calcium chelator EGTA, suggesting that verapamil effect resulted from the inhibition of transmembrane calcium influx. The decrease in apparent proteoglycan synthesis appeared to be attributable primarily to a lowering of the glycanation process, as shown by experiments using an exogenous acceptor for glycosaminoglycan synthesis. Moreover, verapamil induced a decrease in relative proportion of heparan sulfate proteoglycans in the cell layer. Pulse-chase kinetics demonstrated that verapamil also altered proteoglycan catabolism, leading to glycosaminoglycan retention in the cell layer and inhibiting the proteoglycan desulfation step. We conclude that intracellular calcium is essential to maintain Sertoli cell proteoglycan expression and could thus be involved in the repression of Sertoli cell cAMP-dependent syntheses such as estradiol production. J. Cell. Biochem. 76:322–331, 1999.

Key words: rat testis; Sertoli cell; calcium; proteoglycans

Proteoglycans (PG) are complex macromolecules that consist of a protein core to which glycosaminoglycans (GAG) chains are covalently linked [Jackson et al., 1991]. The composition of these chains is based on the repeating dissaccharide unit of one amino sugar and one uronic acid that undergo a series of modification reactions as N- and O-sulfation, N-deacetylation, and epimerization. The extent of these reactions gives rise to large structural heterogeneity [Esko and Zhang, 1996; Salmivirta et al., 1996]. The most prevalent PG contain chondroitin sulfate (CS), dermatan sulfate (DS), or heparan sulfate (HS) GAG chains [Esko, 1991]. PG are widely distributed in vertebrates in the extracellular matrix (ECM), on plasma membranes and within cytoplasmic granules [Wight et al., 1991]. Because of their localization and their ability to interact with a wide range of molecules, PG have been involved in matrix assembly, cell-matrix, and cell-cell adhesion [Iozzo and Murdoch, 1996; Carey, 1997], but they are also important in regulating the morphology, proliferation, and migration of cells [Woods and Couchman, 1996 and 1998].

In the rat testis, the cell types located on both sides of the basement membrane surrounding seminiferous tubules, namely peritubular cells and Sertoli cells, synthesize HSPG and CSPG [Skinner and Fritz, 1985; Mounis et al., 1991; Bichoualne et al., 1994]. PG synthesis has also been observed in interstitial Leydig cells [Grudet et al., 1996]. Cell membrane PG in testicular Sertoli cells have been well structurally characterized [Mounis et al., 1991]. Besides their implication in the above described processes, Sertoli cell PG have been involved in a more differentiated function, as we have shown

*Correspondence to: Gaëlle Fagnen, Laboratoire de Biochimie, UPRES-EA 2608, I.R.B.A., Université de Caen, Esplanade de la Paix, 14032 Caen Cedex, France. E-mail: biochimie@ibba.unicaen.fr

Received 19 April 1999; Accepted 22 July 1999

that alteration of PG synthesis [Phamantu et al., 1995] and sulfation [Phamantu et al., 1999] enhanced follicle-stimulating hormone (FSH)-stimulated estradiol synthesis by regulating phosphodiesterase activity. However, little is known about factors that might regulate their expression in these cells. FSH, the main regulator for the Sertoli cells functions, has no effect on PG synthesis [Skinner and Fritz, 1985], while in granulosa cells, the female counterpart of Sertoli cells, an increase in intracellular cyclic adenosine monophosphate (cAMP), results in stimulation of PG synthesis [Yanagishita et al., 1981]. In addition, with an increase in intracellular cAMP [Leung and Steele, 1992], entry of extracellular calcium has been involved in the signal transduction triggered by FSH in Sertoli cells [Means et al., 1980; Grasso and Reichert, 1989; Gorczynska and Handelsman, 1991]. Whereas cAMP dependence of FSH-induced increase in intracellular calcium remains controversial [Grasso and Reichert, 1990; Gorczynska et al., 1994], it has been clearly demonstrated that calcium influx occurs through voltage-independent and -dependent calcium channels [Grasso and Reichert, 1989; Gorczynska and Handelsman, 1991]. The latter have been identified as both L and N type [Grasso and Reichert, 1989; D'Agostino et al., 1992; Taranta et al., 1997]. Therefore, the aim of the present study was to investigate whether alteration of transmembrane calcium influx induced change in PG production by immature rat Sertoli cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, and trypsin (USP grade) were from Gibco-BRL (Cergy-Pontoise, France). Collagenase-dispase was from Boehringer-Mannheim (Meylan, France). Ultroser SF (Steroid-free serum substitute) and DEAE-Trisacryl were purchased from IBF-Biotechnics (Villeneuve-la-Garenne, France). Superose 6 and FPLC system were provided by Pharmacia Biotech (Saint-Quentin-en Yvelynes, France). Cationic membrane (Zeta-Probe) was from Bio-Rad (Ivry-sur-Seine, France). Bovine pancreas deoxyribonuclease (DNase type I), hyaluronidase (type I-S), Triton X-100, chondroitinase ABC, verapamil, EGTA, A23187, ionomycin, protease inhibitors, Hoechst 33258, and calf thymus DNA were purchased from Sigma (Saint-Quentin-Fallavier, France). [^{35}S]-sulfate (39–59 TBq/mmol) and [^{3}H]-glucosamine (0.7–1.6 TBq/mmol) were provided by NEN (Les Ulis, France). All other reagents were of analytical grade.

Cell Culture

Sertoli cells were isolated from testes of 19- to 21-day-old Sprague-Dawley rats by sequential enzymatic digestion at 32°C as previously described [Tung et al., 1984]. Cells were plated in Falcon 24-multiwell dishes or in Falcon 175-cm^2 flasks at approximately 250,000 cells/cm^2 in Ham's F12-DME medium (1:1) containing 2% Ultroser SF in humidified atmosphere under 5% CO_2 at 32°C. After 48 h, culture medium was removed, and cells were cultured for an additional 24 h in medium without Ultroser SF. On day 3 after plating, residual germ cells were removed by brief hypotonic treatment, using 20 mM Tris-HCl buffer, pH 7.4 [Galdieri et al., 1981]. On day 5, Sertoli cells were labeled for ≤48 h in Ultroser-free culture medium containing carrier-free [^{35}S]-sulfate (10 µCi/ml) and [^{3}H]-glucosamine (2 µCi/ml) in the presence or in absence of treatment.

Quantification of Labeled PG and GAG Synthesized by Rat Sertoli Cells

After labeling, the culture medium was removed and mixed with an equal volume of TUT buffer (100 mM Tris-HCl pH 7, 8 M urea, 1% Triton X-100, protease inhibitors: 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM N-ethylmaleimide, 200 mM 6-aminohexanoic acid, 2 mg/ml pepstatin, 20 mM EDTA, and 10 mM benzamidine-HCl). Sertoli cells in the adherent cell layer were washed three times with 0.01 M phosphate-buffered saline (PBS) and then solubilized with TUT buffer for 24 h at 4°C. TUT buffer was removed and wells were rinsed with the same volume of PBS. Pooled TUT + PBS were then brought to boiling for 10 min.

Quantification of PG from both medium and cell layer extract was carried out by solid-phase assay, using cationic nylon membrane sandwiched in a 96-well dot-blot apparatus (Bio-Rad) as previously described [Rapraeger and Yeaman, 1989]. After laying the samples, the membranes were washed for 30 min with Tris-HCl (50 mM, pH 7) buffer containing 0.15 M NaCl or Tris-HCl buffer, 0.9 M NaCl. The membranes were rinsed and dried in absolute ethanol. Spots were cut and their radioactivity deter-

mined by scintillation counting. Radioactivity in the 0.15 M NaCl-treated dot represents the radioactivity incorporated in PG-associated and free GAG chains, while radioactivity measured in the 0.9 M NaCl-treated dot represents only the radioactivity incorporated in PG-associated GAG chains. For each culture well, radioactivity incorporated in free GAG chains was determined by subtracting the radioactivity of 0.9 M-treated dot from the radioactivity of 0.15 M-treated dot.

For quantitative determination of HSPG, the 0.9 M NaCl-treated nylon blot was treated with nitrous acid specifically in order to hydrolyze heparan sulfate chains [Conrad et al., 1977]. The radioactivity remaining on the membrane represented the nitrous acid-insensitive material, that is, the chondroitin sulfate-containing PG.

Preparation and Characterization of PG-Associated and Free Labeled GAG Chains

The cell layer extract was obtained as mentioned above. To remove the unincorporated radioisotopes and other chemicals, the sample was dialyzed (MW cutoff: 10 kD) against Tris buffer (50 mM, pH 7.2) containing protease inhibitors. [^{3}H]- and [^{35}S]-labeled PG and GAG were then purified by anion-exchange chromatography on a DEAE-Trisacryl column (5 × 1 cm) previously equilibrated with half-concentrated TUT. Elution was performed with 10 ml half-concentrated TUT and then with a linear NaCl gradient (0–1 M) in the same buffer. A flow rate of 15 ml/h was used, and 1-ml fractions were collected. Radioactivity was determined, and the fractions corresponding to the peak of PG and GAG (material eluted between 0.4–0.5 M NaCl) were pooled. After addition of chondroitin sulfate (250 µg/ml), hyaluronic acid (100 µg/ml) and saturated sodium acetate solution (100 µl), samples were precipitated overnight with 5 vol of ethanol at 4°C and centrifuged (18,000*g* at 4°C for 15 min). The pellet was redissolved in distilled water, aliquoted (50,000–100,000 dpm), and lyophilized, and samples were reconstituted with different buffers depending on subsequent treatment. One aliquot (native material) was only dissolved in half-concentrated TUT buffer. To digest chondroitin sulfate chains, a second aliquot was resuspended in 250 µl Tris buffer (50 mM, pH 8.0) containing 0.05 M NaCl, 0.06 M CH_3CO_2Na, 1 mM NaF, bovine serum albumin (BSA)(0.1 mg/ml) and 0.25 U chondroitinase ABC and incubated at 37°C for 24 h [Kleine and Merten, 1981]. In a third aliquot, heparan sulfate chains were degraded by deaminative cleavage using the pH 1.5/HNO_2 method [Conrad et al., 1977]. Aliquots were centrifuged (10,000*g* for 10 min); resulting supernatants were then layered on gel filtration on a Superose 6 column (1 × 30 cm) previously equilibrated with half-concentrated TUT. The elution was carried out with the same buffer at a flow rate of 12 ml/h. Radioactivity present in each fraction (0.4 ml) was determined.

Pulse-Chase Experiments

Confluent Sertoli cells in 24-well culture dishes were double labeled with 10 µCi/ml [^{35}S]-sulfate and 2 µCi/ml [^{3}H]-glucosamine, for 24 h at 32°C. The medium was then removed, and the cell layer was washed extensively with Ham's F12-DME medium to remove free labeled precursors. Labeled Sertoli cells were then chased in fresh culture medium at 32°C for 3, 6, 12, and 24 h, in either the presence or the absence of verapamil. After each chase period, proteoglycans from medium and cell layer were extracted and quantified on cationic membrane as previously described.

Glycosaminoglycan Chain Synthesis

To assess the effects of verapamil on GAG chain synthesis, cell cultures were treated with 1 mM para-nitrophenyl-β-D-xyloside (PNPX) [Carey, 1991] for 24 h, in either the presence or the absence of verapamil and simultaneously double labeled with [^{3}H]-glucosamine and [^{35}S]-sulfate. GAG and PG were then quantified as previously described.

DNA Measurements

DNA content of the cell layer at the end of incubation was quantified by the method of West et al. [1985]. Briefly, Sertoli cell layer was solubilized in NaOH (1 M) and neutralized with KH_2PO_4 (1 M). DNA was quantified in Kontron spectrofluorometer with Hoechst 33258 as fluorescent probe and calf thymus DNA as standard. At the end of incubation, based on a mean DNA content of 6 pg/cell, about 300,000 Sertoli cells were present in each culture well. Variations of cell number were less than 10% between wells and did not exceed 20% between cultures. Thus, because of the reduced ampli-

tude of variations between cell cultures, no attempt was made to expressed radioactivity incorporation as dpm per DNA content.

Expression of Results and Statistics

For quantitative experiments, results were expressed as dpm/500,000 seeded cells or as a percentage of mean value of untreated cultures. In qualitative experiments, the radioactivity present in each elution fraction was expressed as the percentage of total radioactivity eluted from the column. Statistical significance between treatments was determined by Student's *t*-test, using the Statworks package (Cricket Software, Philadelphia, PA) on a Macintosh computer. Differences were considered significant at $P < 0.05$.

RESULTS

Concentration- and Time-Dependent Effect of Verapamil on [^{35}S]-Sulfate and [^{3}H]-Glucosamine Incorporation Into Proteoglycans

In order to study the possible involvement of a calcium influx on PG synthesis, Sertoli cell cultures were treated with increasing verapamil concentrations. A dose-dependent decrease in both [^{3}H]-glucosamine and [^{35}S]-sulfate incorporation in culture medium PG (CM-PG) and cell-associated (CA-PG) was observed (Fig. 1). This decrease was statistically significant when 25 µM verapamil was used (−≥25%). The optimal effect on CM-PG was observed with 150 µM, leading to a 80% decrease in both [^{35}S]- and [^{3}H]-labeling. At 100 µM, [^{3}H]- and [^{35}S]-CA-PG were decreased by 50% and 63% respectively (Fig. 1).

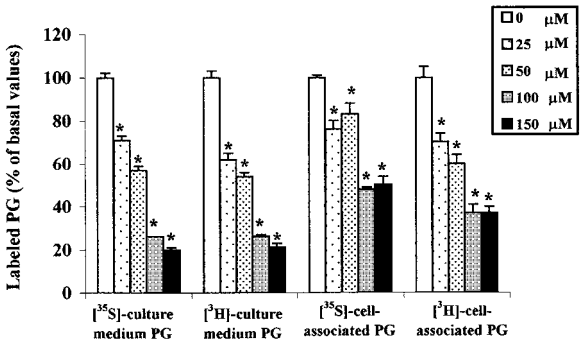

Fig. 1. Dose-dependent effect of verapamil on secreted and cell-associated [^{3}H]- and [^{35}S]-labeled proteoglycans synthesized by cultured rat Sertoli cells. Sertoli cells were incubated for 24 h with [^{35}S]-sulfate (10 µCi/ml) and [^{3}H]-glucosamine (2 µCi/ml) in the presence of increasing concentrations of verapamil. Labeled proteoglycans were quantified by solid-phase assay. Results were expressed as percentage of mean value of untreated cultures in each experiment, the mean ±SEM of four experiments performed in triplicate. Mean basal values were 3240 ([^{35}S]-culture medium PG), 3225 ([^{3}H]-culture medium PG), 588 ([^{35}S] cell-associated PG), 785 ([^{3}H] cell-associated PG) dpm/500,000 cells. *Significantly different (at least $P < 0.05$) from untreated Sertoli cells .

Whatever the concentration used, the DNA content in control and verapamil-treated cultures was similar, indicating that inhibition of PG did not result from cell removal (1,850 ± 211 and 1,790 ± 367 ng DNA/well for control and 100 µM verapamil, respectively).

Kinetic study showed that verapamil-induced decrease in PG labeling was observed from 12 h up to 48 h of incubation (Fig. 2). The most significant effect on [^{3}H]-CA-PG was obtained after 24 h of incubation (−60%), whereas 48 h was necessary for maximal [^{3}H]-CM-PG inhibition (−90%); similar results were obtained with [^{35}S] labeling (data not shown). As many roles have been attributed to CA-PG, further experiments focused on these glycoconjugates.

Calcium Influx Involvment

To determine whether verapamil-induced inhibition of CA-PG labeling resulted from an impaired calcium influx, Sertoli cell cultures were performed in the presence of EGTA in order to chelate extracellular calcium and therefore to reduce its availability. Similar to verapamil, a significant ($P < 0.05$) decrease in CA-PG was observed for 1.06 mM EGTA (437 ± 4 and 313 ± 38 dpm/500,000 cells for control and 1.06 mM EGTA, respectively, in [^{35}S] labeling). When 2 mM extracellular calcium (the concentration found in F12:DME (1:1) is 1.1 mM) was added to EGTA-containing culture medium, EGTA-induced inhibition of CA-PG labeling was abolished (481 ± 28 dpm/500,000 cells in [^{35}S] labeling), suggesting that EGTA effect resulted from decrease in intracellular calcium concentration consecutively to reduced extracellular calcium availability. Similar results were obtained when [^{3}H] labeling was considered (data not shown).

Qualitative Effect of Verapamil on Cell-Associated PG and GAG

Nitrous acid treatment before quantification of sulfated macromolecules by solid-phase assay indicated that HSPG accounted for 74% and 59% of [^{35}S]- and [^{3}H]-labeled CA-PG in untreated Sertoli cells, respectively (Table I). Comparison of the [^{3}H]/[^{35}S] ratio showed that

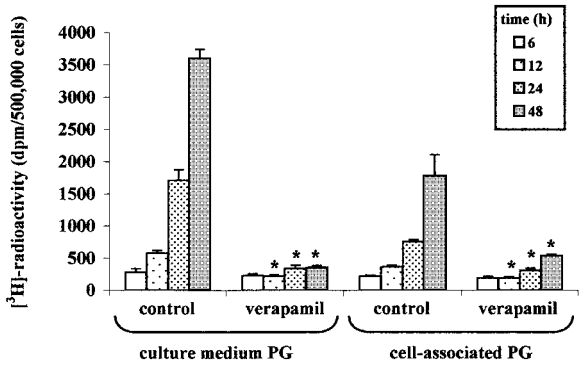

Fig. 2. Time-dependent effect of verapamil on [^{3}H]-labeled secreted and cell-associated proteoglycans synthesized by cultured rat Sertoli cells. Cells were cultured during 6, 12, 24, and 48 h in the absence or presence of 100 μM verapamil. [^{3}H]-labeled proteoglycans were quantified by solid-phase assay. Values were mean ±SEM of triplicate incubations. *Significantly different (at least $P < 0.05$), as compared with their respective control.

CSPG are less sulfated than HSPG (1.41 vs 0.71).

As shown in Table I, verapamil-induced decrease in CA-PG resulted from inhibition of both CS and HS labeling. However, a more pronounced effect was recorded for HSPG (−44%) than for CSPG (−27%). Calculation of [^{3}H]/[^{35}S] ratio showed that the effect of verapamil was not associated with change in sulfation degree (1.39 vs 1.41 and 0.79 vs 0.71 for CSPG and HSPG, respectively).

To characterize CA-PG and CA-GAG altered by verapamil, chromatography was performed as described under Materials and Methods.

TABLE I. Inhibition and Distribution of Cell-Labeled CSPG and HSPG in Verapamil (100 μM)-Treated Sertoli Cell Cultures

Inhibition/ Distribution (%)		HSPG	CSPG
Inhibition[a]			
[^{35}S]-sulfate	Verapamil	−45 ± 3	−28 ± 3
[^{3}H]-glucosamine	Verapamil	−43 ± 9	−26 ± 4
Distribution[b]			
[^{35}S]-sulfate	Control	74 ± 4	26 ± 2
	Verapamil	69 ± 2	31 ± 1
[^{3}H]-glucosamine	Control	59 ± 1	41 ± 2
	Verapamil	53 ± 2	47 ± 3

[a]Mean percentage of inhibition vs. untreated cultures values (488 and 350 dpm/500,000 cells for [^{35}S]- and [^{3}H]-HSPG and 181 and 246 dpm/500,000 cells for [^{35}S]- and [^{3}H]-CSPG respectively).

[b]Values were expressed as relative percentage of total cell-associated PG.

Three distinct labeled peaks were obtained from untreated cultures, exhibiting respective K_{av} of 0.03, 0.21, and 0.53 (Fig. 3). Each population accounted for one-third of total labeled material and, by comparison with the results obtained by Uhlin-Hansen and Yanagishita [1993, 1995], may correspond to large hydrodynamic size PG (peak I), small hydrodynamic size molecules (peak II: small native form, elaborating PG and/or partially degraded PG) and GAG (peak III). The addition of 100 μM of verapamil for 24 h to Sertoli cells induces striking changes in eluted labeled macromolecules (Fig. 3), as reflected by decrease in peak I and shift of peak III toward smaller hydrodynamic size species (K_{av} approximately 0.7). Consequently, the relative percentage of high-molecular-weight molecules was reduced (Table II).

The different populations of macromolecules from untreated and treated cell extracts were analyzed for both their HS and CS content, using chondroitinase ABC digestion and nitrous acid treatment. Table II summarizes the percentage of HS chains present in each population. Whereas HS was the predominant GAG found in all populations, its relative proportion was modified significantly by verapamil. First, HS chains borne by PG eluted with a K_{av} of approximately 0.03 appeared more sulfated (81% vs 75%). Second, HS chains were reduced in peak II (50% vs 61% and 39% vs 63% for [^{35}S]-and [^{3}H] labeling, respectively) and III (61% vs 71% and 60% vs 71% for [^{35}S] and [^{3}H] labeling, respectively). Macromolecules eluted

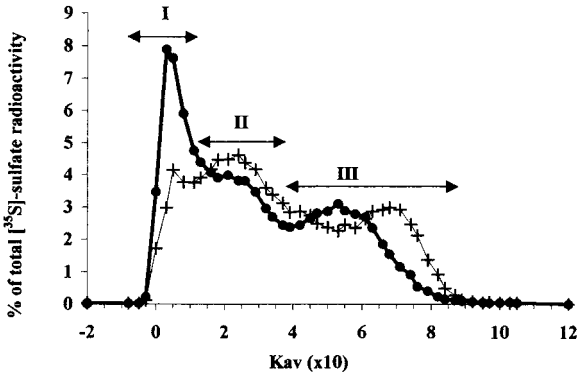

Fig. 3. Size exclusion chromatography of [^{35}S]-labeled cell proteoglycans/glycosaminoglycans (PG/GAG) synthesized in untreated (●) and verapamil-treated (+) Sertoli cells cultures. After purification on DEAE-Trisacryl column, samples (50,000–80,000 dpm) were layered on a Superose 6 column. Radioactivity in each eluted fraction was expressed as percentage of total radioactivity layered on the Superose 6 column.

TABLE II. Qualitative Effect of Verapamil on Macromolecules Populations Issued From Size-Exclusion Chromatography of Sertoli Cell-Associated PG and GAG

	[^{35}S]-sulfate		[^{3}H]-glucosamine	
	Untreated cells	Verapamil-treated cells	Untreated cells	Verapamil-treated cells
Percentage of total PG/GAG[a]				
Peak I	33	16	33	27
Peak II	33	40	33	36
Peak III	33	43	33	39
Percentage of HS[b]				
Peak I	75	81	79	79
Peak II	61	50	63	39
Peak III	71	61	71	60

[a]Percentage of total radioactivity layered on Superose 6 column.
[b]Percentage of HS was mean of nitrous acid-sensitive and chondroitinase ABC-resistant material.

with K_{av} approximately 0.7, probably representing catabolism products issued from PG (peaks I and II), were oversulfated compared with the GAG population (peak III) from control cells ([^{3}H]/[^{35}S] ratio 0.6 vs 1).

PG Turnover in Cultured Rat Sertoli Cells

Cultures were metabolically radiolabeled with [^{3}H]-glucosamine and [^{35}S]-sulfate for 24 h. The fate of labeled cell-associated-PG and -GAG was monitored. As reported in Figure 4, the half-life of labeled CA-PG in untreated cultures was 5–6 h for [^{35}S]-sulfate activity (Fig. 4A) and 9 –12 h for [^{3}H] labeling (Fig. 4B). After 24 h of chase, 20% of [^{35}S]-macromolecules were still present in the cell layer, while 40% were released into medium. The remainder (40%), considered as free [^{35}S]-sulfate, was not recovered as PG or GAG molecules and corresponded probably to free [^{35}S]-sulfate and/or oligosaccharides. By contrast, the totality of [^{3}H] labeling was recovered in macromolecules after 24 h either in medium (70%) and in the cell layer (30%).

The release in culture medium of [^{3}H]-CA-PG from control and verapamil-treated cells proceeded at about the same rate ($t_{1/2}$ = 9 h), whereas the loss of [^{35}S]-sulfate from cell was decreased in a dose-dependent manner by verapamil (data not shown) as reflected by the increase in the half-life of [^{35}S]-PG (7 h and 12 h for 50 and 100 µM verapamil, respectively). Consequently, the recovery of [^{35}S] labeling as free labeled precursor and/or oligosaccharides was drastically reduced (Table III). In addition, at the end of the 24-h chase period, an increase

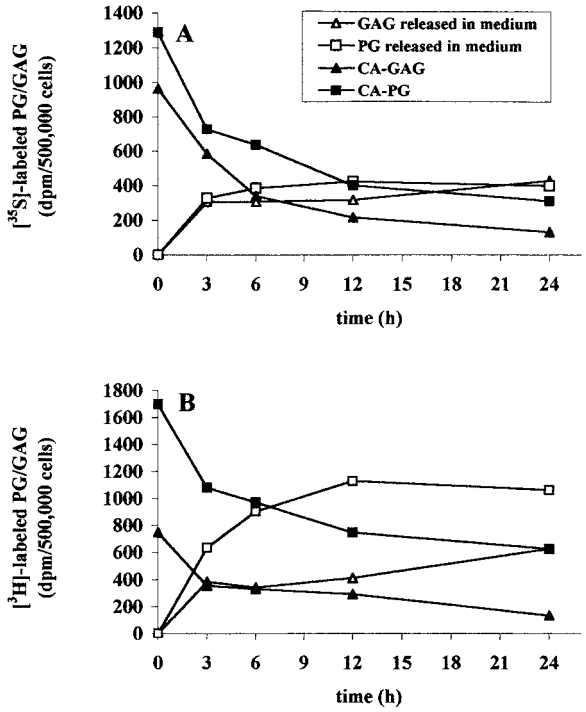

Fig. 4. Pulse-chase kinetics of labeled proteoglycans (PG) and glycosaminoglycans (GAG) in untreated Sertoli cells. Sertoli cells were labeled for 24 h with [^{3}H]-glucosamine and [^{35}S]-sulfate and chased, as described under Materials and Methods. [^{35}S]-sulfate (**A**) and [^{3}H]-glucosamine (**B**) radioactivity's incorporated in cell-associated proteoglycans (CA-PG) and glycosaminoglycans (CA-GAG) and in proteoglycans and glycosaminoglycans released in culture medium were then determined for each incubation time, using quantitative solid-phase assay. Results were mean of three wells in a representative experiment. The variation between different determinations did not exceed ±5% of the mean.

TABLE III. Verapamil-Induced Changes in Catabolism Products of [35S]-Labeled Cell-Associated PG and GAG Molecules at the End of a 24-h Chase*

Verapamil concn	0 μM	50 μM	100 μM
Free [35S]-sulfate[a]	37 ± 3	25 ± 2	1 ± 1
[35S]-GAG[b]	8 ± 1	20 ± 2.5	25 ± 3

*Sertoli cells were labeled for 24 h with [35S]-sulfate and chased in absence or in presence of 50 or 100 μM verapamil. Results were expressed as percentage of initial [35S] labeling associated with cell PG and GAG molecules.
[a]Initial [35S] labeling not incorporated into PG and GAG molecules at the end of chase period was considered free sulfate.
[b][35S]-GAG were quantified by solid-phase assay.

in [35S]-GAG remaining in the cell layer was observed (Table III).

PG /GAG Synthesized in the Presence of Verapamil Under PNPX Conditions

To determine whether verapamil regulates the glycosylation process, Sertoli cells were cultured for 24 h with increasing concentrations of verapamil in presence of 1 mM para-nitrophenyl-β-D-xyloside (PNPX), an exogenous primer for GAG chain initiation competing with endogenous xylosylated core proteins. Under these specific culture conditions, availability in GAG chains acceptor was not a limiting step of biosynthesis, and the maximum capacity of enzymes to attach and elongate GAG chains can be evaluated [Carey, 1991]. [35S]-sulfate and [3H]-glucosamine incorporation into neosynthesized PG/GAG molecules was determined by quantitative solid-phase assay, as described under Materials and Methods. As previously shown in Sertoli cells [Phamanthu et al., 1995], after a 24-h incubation, PNPX (1 mM) induced a decrease in total PG synthesis (65%) and, at the same time, a dramatic increase (8000%) in PNPX-GAG.

Simultaneous incubation of Sertoli cells with PNPX and verapamil decreased in a dose-dependent manner incorporation of both labeled precursors in total (cell layer + medium) macromolecules (PG + GAG) (Fig. 5). Slight inhibition (−13%) was obtained with 10 μM, and the maximal effect was reached for 100 μM, leading to about 84% inhibition for [35S] labeling (Fig. 5). Similar results were obtained for [3H] labeling (data not shown). This overall effect was almost exclusively the result of PNPX-GAG inhibition, since PG were significantly decreased only for 100 μM verapamil (−55% for [35S]-sulfate and −38% for [3H]-glucosamine).

To assess that the effect of verapamil resulted from inhibition of transmembrane calcium influx, PNPX experiments were performed in presence of inefficient concentration of verapamil (25 μM) with or without 1.06 mM EGTA. As reported in Table IV, 25 μM verapamil addition has no significant effect on glycanation process. On the contrary, a significant decrease in PNPX-GAG was recorded with 1.06 mM EGTA (−23% and −50% for [35S] and [3H] labeling, respectively). The addition of 25 μM verapamil together with EGTA potentiated the effect of EGTA by further decreasing PG glycanation (−43% and −61% for [35S]- and [3H] labeling, respectively).

DISCUSSION

Our data demonstrate that L-type voltage-operated calcium channel blocker verapamil induces a sharp decrease in PG synthesis, affecting both secreted and cell-associated PG. This dose- and time-dependent effect is not consequential to an alteration in cell number, since DNA content was not modified significantly by verapamil.

The effect of verapamil on Sertoli cell PG synthesis probably results from the decrease in transmembrane calcium influx. Whereas no attempt was made to measure intracellular calcium concentration, the above hypothesis is supported by (1) a similar effect of EGTA, and (2) the reversal of EGTA-induced inhibition by addition of extracellular calcium. As previously

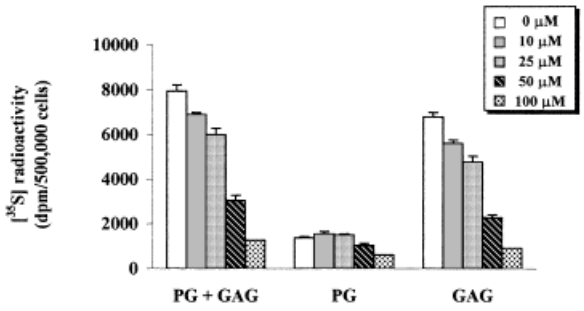

Fig. 5. Effect of verapamil on total synthesis of proteoglycans (PG) and glycosaminoglycans (GAG) under PNPX (1 mM) conditions. Sertoli cells were incubated for 24 h with [35S]-sulfate and 1 mM PNPX in absence or in presence of increasing concentrations of verapamil. Labeled PG and GAG were quantified by solid-phase assay, as described under Materials and Methods. Values are mean ±SEM of three experiments performed in triplicate.

TABLE IV. Effect of 25 μM Verapamil and 1.06 mM EGTA, Combined or Alone, on Labeled PG and GAG Synthesized by Rat Sertoli Cells Under PNPX Conditions

	[^{35}S]-sulfate		[^{3}H]-glucosamine	
	PG + GAG	GAG	PG + GAG	GAG
PNPX (1 mM)	7,070 ± 140[a]	5,460 ± 200[a]	5,330 ± 260[a]	4,460 ± 330[a]
+verapamil (25 μM)	6,750 ± 420[a]	5,230 ± 360[a]	4,580 ± 570[a]	3,620 ± 620[a]
+EGTA (1.06 mM)	5,240 ± 370[a,b]	4,180 ± 270[a,b]	2,840 ± 500[a,b]	2,240 ± 480[a,b]
+verapamil + EGTA	4,380 ± 180[a,b]	3,090 ± 140[a,b]	2,440 ± 110[a,b]	1,730 ± 120[a,b]

[a]Values were expressed as dpm/500,000 cells.
[b]Values were significantly different at $P < 0.05$ from values without the [b] superscript in the same column.

shown by Gorczynska and Handelsman [1991], intracellular calcium concentration in Sertoli cells is highly dependent on concentration and/or availability of extracellular concentration. Thus, the effect of verapamil on PG synthesis results from decrease in transmembrane calcium flux. Intracellular calcium concentration either stimulates PG synthesis in bovine granulosa cells [Lenz et al., 1982; Bellin et al., 1983] and in breast cancer cells [Vandewalle et al., 1994] or decreases PG synthesis in chondrocytes [Eilam et al., 1985] and parathyroid cells [Takeushi et al., 1990; Muresan and MacGregor, 1994].

Qualitative studies issued from both solid-phase assay and chromatographic experiments showed that verapamil inhibits labeling of both CS- and HS-GAG chains linked to Sertoli cell-associated PG, with more pronounced effect on HS chains. Furthermore, the addition of verapamil to Sertoli cell cultures leads to a shift from large hydrodynamic size molecules (K_{av} 0.03/0.05) toward small hydrodynamic size (K_{av} = 0.24). Moreover, it promotes appearance of small HS-rich molecules (K_{av} = 0.68). Whether this population results from increase in catabolism of high hydrodynamic-size population induced by verapamil is very likely but remains to be demonstrated. In spite of changes recorded in individual populations, verapamil does not induce any modification of the overall sulfation of macromolecules, as it decreases in the same extent both [^{3}H]- and [^{35}S]-sulfate labeling of cell-associated PG.

PG biosynthesis is a complex process involving first-core protein elaboration followed by initiation and elongation of the GAG chain. As shown in experiments using PNPX, calcium is involved in the regulation of glycanation process in Sertoli cells. Under PNPX conditions (i.e., when endogenous core protein is not a rate-limiting step of PG synthesis), the addition of verapamil decreases labeling of neosynthesized GAG primed on PNPX in a dose-dependent fashion. Because of the same magnitude of response to verapamil, whether under PNPX conditions or not, the glycanation process is probably the main step of PG synthesis affected by verapamil. Because verapamil inhibits both CS and HS GAG chains, it may be suggested that verapamil alters one or more types of early enzymatic activity of the glycanation process, common to both CS and HS synthesis, that is, galactosyltransferase I and II or glucuronosyltransferase. The calcium requirements for galactosyltransferase I and II are unknown, but it is known that calcium is required for higher glucuronosyltransferase activity in vitro [Lidholt and Lindahl, 1992].

PNPX experiments, exhibiting both similar effects of EGTA and verapamil and potentiation of inefficient concentration of verapamil by EGTA, suggest that inhibition of glycanation results from inhibition of transmembrane calcium influx and, subsequently from decrease in intracellular calcium concentration. Despite the sharp effects of verapamil on glycanation process, which are sufficient to explain decrease in PG production, we cannot exclude a minor effect of calcium on the initial steps of anabolic process (nucleotide sugars synthesis and transport, xylosyltransferase activity, or core protein synthesis).

In contrast with ovarian granulosa cells [Yanagishita, 1992] and other types of cells [Silbert and Sugumaran, 1995], the main route of Sertoli cell PG catabolism is the shedding from cell surface into medium and subsequent catabolism into smaller molecules, as shown by pulse-chase experiments. Verapamil does not modify the rate of shedding process (as reflected by [^{3}H]-labeled PG released from cell membrane), but it does change the fate of [^{35}S] labeling since, in contrast with untreated cells,

[^{35}S] radioactivity remains associated with PG and GAG molecules. The results of pulse-chase experiments show that (1) desulfation of cell PG, which occurs before or simultaneous to their release into culture medium, is decreased by verapamil; and (2) verapamil induces cell retention of sulfated GAG, as shown by an increase in [^{35}S]-labeled CA-GAG in verapamil-treated cultures and by the appearance of a small hydrodynamic size population of oversulfated GAG (K_{av} = 0.7, Fig. 3) on the chromatographic profile. Whether the GAG retention in verapamil-treated cell layer results from an increase in intracellular heparanase activity or from an inhibition of GAG released into medium, or both, remains to be determined. The intracellular location of heparanases [Nakajima et al., 1986] and the reduced susceptibility of undersulfated HSPG to these enzymes [Bame, 1993; Bai et al., 1997] favor the first hypothesis. This finding suggests that a decrease in cell PG desulfation in verapamil-treated cells, thereby reducing their shedding into medium, favors PG degradation by intracellular heparanases.

The physiological significance of the calcium regulation of Sertoli cell PG metabolism remains to be elucidated. Experiments using EGTA, verapamil, or calcium ionophore [Malléa et al., 1987; Grasso and Reichert, 1989; Talbot et al., 1991] clearly indicate that calcium antagonizes the stimulatory effect of FSH on cAMP production and aromatase activity in Sertoli cells by an unknown mechanism. In addition, we have previously demonstrated that cellular HSPG level is inversely correlated with FSH-stimulated estradiol production [Phamanthu et al., 1995, 1999]. These data, taken together with our present study, might suggest that a decrease in cell HSPG content induced by calcium flux inhibition is an additional mechanism by which verapamil (or EGTA) induces enhancement of steroidogenesis. At this time, our attempts to modify PG synthesis by increasing intracellular calcium levels, either by adding exogenous calcium or by using calcium ionophore (A23187 or ionomycin) proved unsuccessful. At concentrations commonly used in the literature, these molecules lead to subsequent cell death in our culture conditions (data not shown). Moreover, lower concentrations are inefficient in modifying Sertoli cell PG synthesis. Nevertheless, the calcium influx induced by FSH in Sertoli cells [Gorczynska and Handelsman, 1991] could serve at least to maintain and eventually increase Sertoli cell membrane HSPG expression, thus reducing the FSH responsiveness of Sertoli cells.

REFERENCES

Bai X, Bame KJ, Habuchi H, Kimata K, Esko JD. 1997. Turnover of heparan sulfate depends on 2-O-sulfation of uronic acids. J Biol Chem 272:23172–23179.

Bame KJ. 1993. Release of heparan sulfate glycosaminoglycans from proteoglycans in Chinese Hamster Ovary does not require proteolysis of the core protein. J Biol Chem 268:19956–19964.

Bellin ME, Lenz RW, Steadman LE, Ax RL. 1983. Proteoglycan production by bovine granulosa cells in vitro occurs in response to FSH. Mol Cell Endocrinol 29:51–65.

Bichoualne L, Thiébot B, Langris M, Barbey P, Oulhaj H, Bocquet J. 1994. Membrane associated proteoglycans in rat testicular peritubular cells. Mol Cell Biochem 140: 37–48.

Carey DJ. 1991. Biological functions of proteoglycans: use of specific inhibitors of proteoglycan synthesis. Mol Cell Biochem 104:21–28.

Carey DJ. 1997. Syndecans: multifunctional cell-surface co-receptors. Biochem J 327:1–16.

Conrad GW, Hamilton C, Haynes E. 1977. Differences in glycosaminoglycans synthesized by fibroblast-like cells from chick cornea, heart, and skin. J Biol Chem 252:6861–6870.

D'Agostino A, Mene P, Stefanini M. 1992. Voltage-gated calcium channels in rat Sertoli cells. Biol Reprod 46:414–418.

Eilam Y, Beit-Or A, Nevo Z. 1985. Decrease in cytosolic free Ca^{2+} and enhanced proteoglycan synthesis induced by cartilage derived growth factors in cultured chondrocytes. Biochem Biophys Res Commun 132:770–779.

Esko JD. 1991. Genetic analysis of proteoglycan structure, function and metabolism. Curr Opin Cell Biol 3:805–816.

Esko JD, Zhang L. 1996. Influence of core protein sequence on glycosaminoglycan assembly. Curr Opin Struct Biol 6:663–670.

Galdieri M, Ziparo E, Palombi F, Russo MA, Stefanini M. 1981. Pure Sertoli cell: a new model for the study of somatic germ cell interactions. J Androl 5:249–254.

Gorczynska E, Handelsman DJ. 1991. The role of calcium in follicle-stimulating hormone signal transduction in Sertoli cells. J Biol Chem 266:23739–23744.

Gorczynska E, Spaliviero J, Handelsman DJ. 1994. The relationship between 3′,5′-cyclic adenosine monophosphate and calcium in mediating follicle-stimulating hormone signal transduction in Sertoli cells. Endocrinology 134:293–300.

Grasso P, Reichert LE Jr. 1989. Follicle-stimulating hormone receptor-mediated uptake of $^{45}Ca^{2+}$ by proteoliposomes and cultured rat Sertoli cells: evidence for Involvment of voltage-activated and voltage-independent calcium channels. Endocrinology 125:3029–3036.

Grasso P, Reichert LE Jr. 1990. Follicle-stimulating hormone receptor-mediated uptake of Ca^{2+} by cultured rat Sertoli cells does not require activation of cholera toxin- or pertussis toxin-sensitive guanine nucleotide binding proteins or adenylate cyclase. Endocrinology 127:949–956.

Grudet N, Bonnamy PJ, Bocquet J, Carreau S. 1996. Proteoglycan synthesis by purified Leydig cells from immature and mature rats. CR Acad Sci Paris 319:1101–1105.

Iozzo RV, Murdoch A. 1996. Proteoglycans of the extracellular environment: clues from the gene and protein side offer novel perspectives in molecular diversity and function. FASEB J 10:598–614.

Jackson RL, Busch SJ, Cardin A. 1991. Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes. Physiol Rev 71:481–539.

Kleine TO, Merten B. 1981. A procedure for the simultaneous determination of small quantities of hyaluronate and isomeric chondroitin sulfate by chondroitinases. Anal Biochem 118:185–190.

Lenz RW, Ax RL, First NL. 1982. Proteoglycan production by bovine granulosa cells in vitro is regulated by calmodulin and calcium. Endocrinology 110:1052–1054.

Leung PCK, Steele GI. 1992. Intracellular signaling in the gonads. Endocr Rev 13:476–498.

Lidholt K, Lindahl U. 1992. Biosynthesis of heparin. The D-glucuronosyl- and N-acetyl-D-glucosaminyltransferase reactions and their relation to polymer modifications. Biochem J 287:21–29.

Malléa LE, Machado AJ, Navaroli F, Rommerts FFG. 1987. Modulation of stimulation action of follicle stimulating hormone (FSH) and inhibitory action of epidermal growth factor (EGF) on aromatase activity in Sertoli cells by calcium. FEBS Letters 218:143–147.

Means AR, Dedman JR, Tash JS, Tindall DJ, van Sickle M, Welsh MJ. 1980. Regulation of the testis Sertoli cell by follicle stimulating hormone. Annu Rev Physiol 42:59–70.

Mounis A, Barbey P, Langris M, Bocquet J. 1991. Detergent-solubilized proteoglycans in rat testicular Sertoli cells. Biochim Biophys Acta 1074:424–432.

Muresan Z, MacGregor R. 1994. The release of parathyroid hormone and the exocytosis of a proteoglycan are modulated by extracellular Ca^{2+} in a similar manner. Mol Biol Cell 5:725–737.

Nakajima M, Irimura T, Nicolson GL. 1986. A solid-phase substrate of heparanase: its application to assay of human melanoma for heparan sulfate degradative activity. Anal Biochem 157:162–171.

Phamantu NT, Bonnamy PJ, Bouakka M, Bocquet J. 1995. Inhibition of proteoglycan synthesis induces an increase in follicle stimulating hormone (FSH)-stimulated estradiol production by immature rat Sertoli cells. Mol Cell Endocrinol 109:37–45.

Phamantu NT, Fagnen G, Godard F, Bocquet J, Bonnamy PJ. 1999. Sodium chlorate induces undersulfation of cellular proteoglycans and increases in FSH-stimulated estradiol production in immature rat Sertoli cells . J Androl 20:141–150.

Rapraeger A, Yeaman C. 1989. A quantitative solid-phase assay for identifying radiolabeled glycosaminoglycans in crude cell extracts. Anal Biochem 179:361–365.

Salmivirta M, Lidholt K, Lindahl U. 1996. Heparan sulfate: a piece of information. FASEB J 10:1270–1279.

Silbert JE, Sugumaran G. 1995. Intracellular membranes in the synthesis, transport, and metabolism of proteoglycans. Biochim Biophys Acta 1241:371–384.

Skinner MK, Fritz IB. 1985. Structural characterization of proteoglycans produced by testicular peritubular cells and Sertoli cells. J Biol Chem 21:11874–11883.

Takeuchi Y, Sakagushi K, Yanagishita M, Aurbach GD, Hascall VC. 1990. Extracellular calcium regulates distribution and transport of heparan sulfate proteoglycans in rat parathyroid cell line. J Biol Chem 265:13661–13668.

Talbot JA, Lambert A, Mitchell R, Grabinski M, Anderson DC, Tsatsoulis A, Shalet SM, Robertson WR. 1991. FSH-dependent estrogen secretion by rat Sertoli cells in vitro: modulation by calcium. Acta Endocrinol 125:280–285.

Taranta A, Morena AR, Barbacci E, D'Agostino A. 1997. ω-Conotoxin-sensitive Ca^{2+} voltage-gated channels modulate protein secretion in cultured rat Sertoli cells. Mol Cell Endocrinol 126:117–123.

Tung PS, Skinner MK, Fritz IB. 1984. Fibronectin synthesis is a marker for peritubular cell contaminants in Sertoli cell enriched cultures. Biol Reprod 30:199–211.

Uhlin-Hansen L, Yanagishita M. 1993. Differential effect of brefeldin A on the biosynthesis of heparan sulfate and chondroitin/dermatan sulfate proteoglycans in rat ovarian granulosa cells in culture. J Biol Chem 268:17370–17376.

Uhlin-Hansen L, Yanagishita M.1995. Brefeldin A inhibits the endocytosis of plasma-membrane-associated heparan sulfate proteoglycans of cultured rat ovarian granulosa cells. Biochem J 310:271–278.

Vandewalle B, Revillion F, Hornez L, Lefebvre J. 1994. Calcium regulation of heparan sulfate proteoglycans in breast cancer cells. J Cancer Res Clin Oncol 120:389–392.

West DC, Sattar A, Kumar S. 1985. A simplified in situ solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. Anal Biochem 147:289–295.

Wight TN, Heinegard DK, Hascall VC. 1991. Proteoglycans: structure and function. In: Hay ED, editor. Cell biology of extracellular matrix. New York: Plenum. p 45–78.

Woods A, Couchman JR. 1996. Signaling from the matrix to the cytoskeleton: role of cell surface proteoglycans in matrix assembly. Kidney Int 49:S64–S67.

Woods A, Couchman JR. 1998. Syndecans: synergistic activators of cell adhesion. Trends Cell Biol 8:189–192.

Yanagishita M. 1992. Glycosylphosphatidylinositol-anchored and core protein-intercalated heparan sulfate proteoglycans in rat ovarian granulosa cells have distinct secretory, endocytotic, and intracellular degradative pathways. J Biol Chem 267:9509–9511.

Yanagishita M, Hascall VC, Rodbard D. 1981. Biosynthesis of proteoglycans by rat granulosa cells cultured in vitro: modulation by gonadotropins, steroid hormones, prostaglandins, and a cyclic nucleotide. Endocrinology 109: 1641–1649.